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Effects of Prenatal Testosterone on the Reproductive and Metabolic Neurons of the Sheep Hypothalamus

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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(Thesis Format: Integrated Article)

by

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Abstract

Polycystic ovary syndrome (PCOS) has been recognized as one of the most common endocrine disorders affecting reproductively aged women. PCOS is a complex disorder of developmental origin comprising of genetic and environmental factors, including abnormal exposure to androgens during a critical period of prenatal development. As such, women with PCOS and ewes prenatally exposed to testosterone (T) show similar reproductive and metabolic defects, including anovulatory dysfunctions stemming from abnormal gonadotropin releasing hormone GnRH/luteinizing hormone LH secretion, insulin resistance, and hyperinsulinemia. Our lab has previously shown that prenatal T-treated sheep show alterations in arcuate (ARC) KNDy (co-expressing kisspeptin, neurokinin B/dynorphin) and agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC), two populations that play an important role in the control of reproduction and metabolism, respectively. For this dissertation, I examine the effects of prenatal T treatment on androgen receptor (AR) and insulin receptor (IR) expression with the reproductive and metabolic neurons of the hypothalamus, the ARC KNDy, AgRP and proopiomelanocortin (POMC) neurons, the preoptic area (POA) kisspeptin neurons, and the GnRH neurons. Analysis of immunohistochemical staining revealed that prenatal T treatment increases AR-immunoreactivity (ir) in several brain areas of the sheep brain as well as colocalization of AR with ARC KNDy and AgRP and POA kisspeptin neurons. Conversely, prenatal T treatment decreases IR colocalization with ARC KNDy and AgRP neurons, but not POMC neurons, while POA kisspeptin and GnRH neurons do not colocalize IR. Prenatal, but not postnatal interventions blocking androgen action (Flutamide, F) or increasing sensitivity to insulin (Rosiglitazone, R) can prevent the

prenatal T induced alterations in these populations. Lastly, I show that leptin, an important metabolic signal that can influence GnRH secretion and reproductive function, indirectly activates ARC KNDy neurons through a pathway that may include the leptin-sensitive AgRP and POMC neurons. Overall, these results indicate that prenatal exposure to T masculinizes the female brain and alters insulin signalling in the reproductive and metabolic neurons of the ARC and highlights the importance of prenatal interventions in blocking these effects. These changes may contribute the reproductive and metabolic dysfunctions associated with the PCOS phenotype.

Keywords:

Prenatal testosterone; PCOS; Kisspeptin; KNDy; AgRP; POMC; androgen receptor; insulin receptor; leptin; PCOS; metabolism; reproduction; insulin sensitizer, androgen blocker

Co-Authorship Statement

This dissertation incorporates material that is the result of collaborative research efforts. All contents of the current dissertation were carried out in collaboration with Michael N. Lehman and Lique M. Coolen. In addition, a majority of this work (chapters 2-4) was performed in collaboration with Vasantha Padmanabhan, a long-time collaborator of Drs. Lehman and Coolen. Additionally, the following collaborators have contributed to the completions of particular studies and deserve recognition:

1) Insulin Receptor manuscript (Chapter 3): Rebecca Phillips completed the immunohistochemical staining and analysis of insulin receptor colocalization with AgRP and POMC neurons.

2) Androgen Receptor manuscript (Chapter 2): Brains of ewes prenatally exposed to T, DHT and E and ram brains were perfused and sectioned by Theresa Lee's lab.

3) Leptin manuscript (Chapter 5): Leptin injections were performed by Rebecca Phillips (2011). pSTAT3 staining and analysis (kisspeptin, GnRH and POMC) was also completed by Rebecca Philips.

4) Animal procedures and tissue collection were carried out at the University of Michigan sheep research facility. Care and treatments of animals at the farm was a collaborative effort between the Coolen/Lehman lab, the Padmanabhan lab and the farm personnel (managed by Doug Doop).

In addition to the work described above, I have taken part in animal work at the Sheep Research Facility at the University of Michigan, Ann Arbor, Michigan, and have contributed to numerous research studies at this facility, including brain perfusions and extractions, blood sampling, blood processing, fetal tissue collection surgery, fetal organ preservation, and lambing (ear tagging, weighing and blood sampling).

Acknowledgments

The writing of this dissertation has been a challenging, yet rewarding experience. Without the support patience and guidance of the following people, this study could not have been completed. First and foremost, my utmost recognition goes to my supervisor and mentor, Dr. Michael Lehman, whose encouragement and gratitude I will never forget. Dr. Lehman`s strong leadership and commitment to the highest standards has been my constant inspiration as I hurdle all the obstacles in the completion of this research work. I would also like to express my gratitude to Dr. Lique Coolen, for her excellent guidance and patience throughout the years. Her devotion to research and caring attitude always made me feel like I had strong supporter on my side. Both Dr. Lehman and Dr. Coolen have provided me with an excellent graduate experience, a wonderful working atmosphere and a strong foundation in research.

I would like to thank my committee members, Dr. Arthur Brown, Dr. Steve Laviolette and Dr. Walter Rushlow for their guidance and excellent suggestions, even when our committee meetings changed from being over the table to over the teleconferencing phone.

I would like to acknowledge all the students, volunteers and employees of the Sheep Research Facility in Ann Arbor, Michigan. The extensive research projects would not have been possible without the contribution of these people, who devoted most of their time to take part in my, and many other research projects. Special thanks to Doug Doop, who managed the sheep farm. Doug was always the first one there and often the last to leave, with a smile on his face the entire time. The great times experienced at the farm will always remain a highlight in my research career.

Special thanks to all the members of the Lehman/Coolen lab. Throughout the years, many have come and gone, but each has had an impact in my life in some way or another. The graduate students and post doctorate fellows have always provided me with unconditional support and encouragement. The friendships I have created over the years will always remain in my life and the great times we shared together will always be fondly remembered. I would also like to acknowledge the laboratory technicians who put in endless hours assisting with various projects and always doing it with a smile on their face.

Last, but not least I would like to thank my family and partner for their constant encouragement and positive outlook, which kept me motivated during difficult times. As my greatest inspiration, my mother provided me with endless love and encouragement. She has taught me that with hard work, patience and love, any obstacle can be overcome. My sisters, Elena and Lucia, were my biggest cheerleaders and always kept me going with constant support. Lastly, my partner and best friend, Adam, has been my biggest fan throughout this experience. During the course of my doctoral studies, our lab moved from London, Ontario to Ann Arbor, Michigan and lastly to Jackson, Mississippi. Without the support and encouragement of my family and partner, this process would have been much more difficult.

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List of Abbreviations and Symbols

3V	Third Ventricle
17β HSD	17β-hydroxy steroid dehydrogenase
α	Alpha
β	Beta
γ	Gamma
A	Androstenedione
ABC	Avidin-biotin-HRP conjugate
ac	Anterior commissure
AgRP	Agouti related- peptide
AR	Androgen receptor
ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
BBB	Blood brain barrier
BMI	Body mass index
BNST	Bed nucleus of the stria terminalis
BT	Biotinylated tyramine
C	Control
CART	Cocaine- and amphetamine- regulated transcript
CP	Cerebral peduncle
CSF	Cerebrospinal fluid
DAB	Diaminobenzidine tetrahydrochloride

DHEAS	Dehydroepiandrosterone sulfate
DIO	Diet induced obesity
DMH	Dorsomedial hypothalamus
DYN	Dynorphin
E_2	Estrogen
ER	Estrogen receptor
F	Flutamide
FSH	Follicle stimulating hormone
Foxo1	Forkhead box protein O1
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein
GnRH	Gonadotropin releasing hormone
IgG	Immunoglobulin G
IHC	Immunohistochemistry
ir	Immunoreactivity
IR	Insulin receptor
IRS	Insulin receptor substrate
JAK	Janus kinase
Kiss	Kisspeptin
KNDy	Kisspeptin, Neurokinin B/Dynorphin
KO	Knock-out
LepRa	Leptin receptor a

LepRb	Leptin receptor b
LH	Luteinizing hormone
LHA	Lateral hypothalamic area
MAPK	Mitogen-activated protein kinase
MBH	Mediobasal hypothalamus
MCR	Melanocortin receptor
ME	Median eminence
MSH	Melanocyte stimulating hormone
mt	Mammillothalamic tract
NGS	Normal goat serum
NIRKO	Neuron-specific insulin receptor knockout
NKB	Neurokinin B
NTS	Nucleus of the solitary tract
NPY	Neuropeptide Y
oc	Optic Chiasm
ot	Optic Tract
OVLT	Oragnum vasculosum of lamina terminalis
P	Progesterone
PAG	Periaqueductal gray
PT	Pituitary
pY	phosphor-tyrosine
PB	Phosphate buffer
PBS	Phosphate buffered saline

PCOS	Polycystic ovary syndrome
PI3K	Phosphoinositide-3 kinase
PMd	dorsal premammillary nucleus
PMv	Ventral premammillary nucleus
POA	Preoptic area
POMC	Proopiomelanocortin
PVN	Paraventricular hypothalamic nucleus
qPCR	Real-time quantitative polymerase chain reaction
R	Rosiglitazone
RCA	Retrochiasmatic area
SHBG	Sex hormone binding globulin
SOCS	suppressor of cytokine signalling
SON	Supraoptic nucleus
STAT	Signal transducer and activator of transcription
T	Testosterone
Tyr	Tyrosine
VMH	Ventromedial nucleus of hypothalamus
VTA	Ventral tegmental Area
wt	Wild-type

List of Appendices

Chapter 1: Literature Review

1.1 Regulation of gonadotropin secretion in the normal menstrual cycle

In mammals, ovulatory function relies on a series of complex and integrated interactions between the hypothalamus, pituitary gland and ovaries (Fig. 1). The hypothalamic-pituitary unit is the principal driver of reproductive function. It controls gonadal function by the dual action of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The synthesis and release of these gonadotropins are regulated by gonadotropin releasing hormone (GnRH), released in a pulsatile manner from GnRH neurons in the hypothalamus. The frequency of GnRH pulses largely determines which gonadotropin hormone is synthesized and secreted by the same gonadotrope cell. Rapid GnRH pulses favor LH, whereas slower GnRH pulses favor FSH secretion (*1-2*). In females, two modes of GnRH secretion occur at different times during the ovarian cycle. Through most of the cycle, GnRH is secreted in a pulsatile manner (*3-4*). During this time, estradiol (E_2) and progesterone (P) act in a negative feedback manner to regulate GnRH pulsatile secretion, with E_2 inhibiting pulse amplitude and P inhibiting pulse frequency (*5*). GnRH pulsatility progressively increases during the follicular phase, partly due to a gradual loss of the restrictive influence of progesterone (*6*), reaching a peak of one pulse per hour during the late follicular phase (*7*). At this stage of the cycle, the preovulatory GnRH/LH surge is induced by the positive feedback actions of high concentrations of E_2 produced by the dominant ovarian follicle(s). The

GnRH/LH surge is marked by an upsurge in pituitary LH release in response to the rapid GnRH pulses, which in turn stimulates ovulation (*8*-*9*). Following ovulation, the corpus luteum secretes E_2 and P, which act in a negative feedback manner to slow GnRH pulses to one pulse every 3–4 h (*7*). This pulse frequency favors synthesis and release of FSH and initiation of the next wave of follicular development. Considering the complex set of interactions that are essential for ovulatory function, alterations at any level of the hypothalamic–pituitary–ovarian axis can result in ovulatory dysfunction.

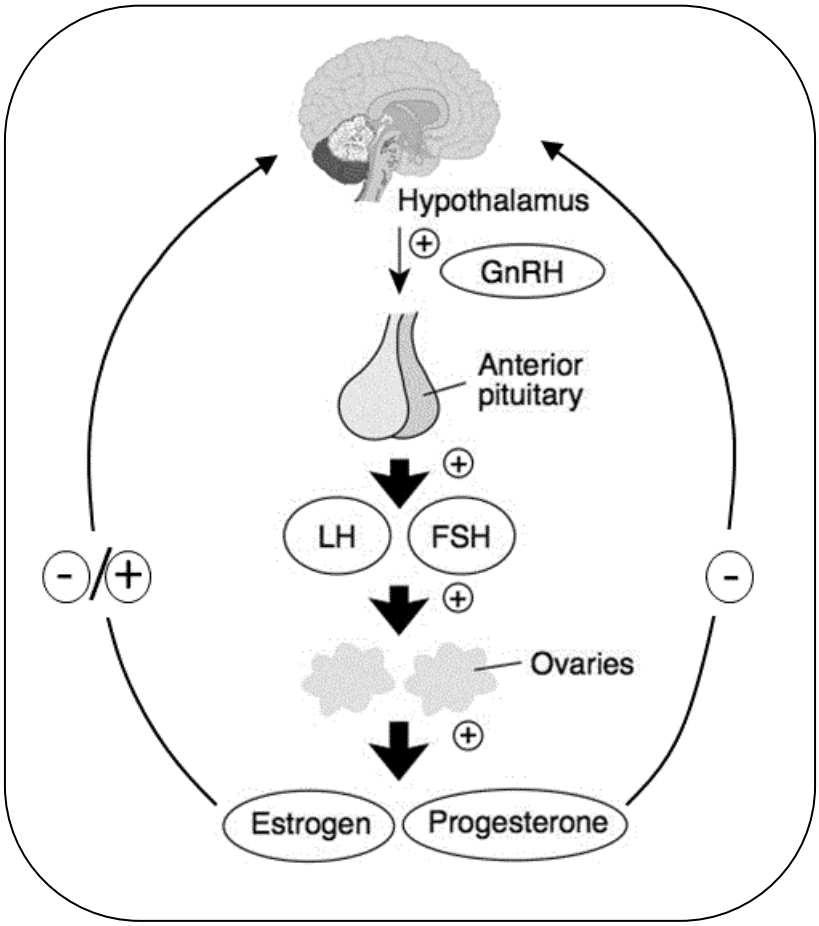

Figure 1. Schematic diagram illustrating the female hypothalamo-pituitary-gonadal (HPG) axis. *Abbreviations*: GnRH: gonadotropin releasing hormone, LH: luteinizing hormone, FSH: follicle stimulating hormone.

1.2 Prenatal programming of reproduction and the origins of polycystic ovarian syndrome (PCOS)

Several decades ago, David Barker proposed that exposure of the developing fetus to certain environmental factors could permanently program physiology function during adulthood (*10*). Among these factors are the gonadal steroid hormones. It is now widely accepted that exposure of the developing female to high levels of androgens during the critical period of prenatal development, permanently changes the organization of the neuroendocrine axis, setting the framework for dysfunctions in adulthood (*11*). The gonadal steroid hormones, testosterone (T) and estrogen (E_2) are important for organizing the brain during early development as well as regulating growth, maintenance and function of various reproductive tissues. In the brain and other tissues, testosterone can be aromatized to estradiol by the enzyme aromatase and act on estrogen receptors (ER) (*12-13*). Alternatively, 5α-reductase can reduce testosterone to more a potent androgen dihydrotestosterone (DHT) which also binds androgen receptors (AR) (*14-15*) (Fig. 2). In humans, a neonatal surge in T starts at about 2 weeks of age and peaks over the next 12–17 weeks (*16*); however, T levels remain approximately ten times lower in female fetuses compared to males (*17*). It has been proposed that the increase in T is critical for sexual differentiation of the brain, specifically masculinization and defeminization in the male (*18-19*). As such, exposure of the female fetus to excess T during critical periods of prenatal development results in phenotypic, behavioural and reproductive abnormalities in humans (*20-21*). This has also been documented in other mammalian species including monkeys (*22-23*), sheep (*24-27*), rats (*28*), mice (*29*), and guinea pigs (*30*). This type of

abnormal steroid exposure can occur when female fetuses (or their mothers) produce excessive levels of androgens (ie. congenital adrenal hyperplasia (CAH) (*31*) or are exposed to androgens via an exogenous source, such as endocrine disruptive agents (*32-33*) or certain medical interventions (*34*).

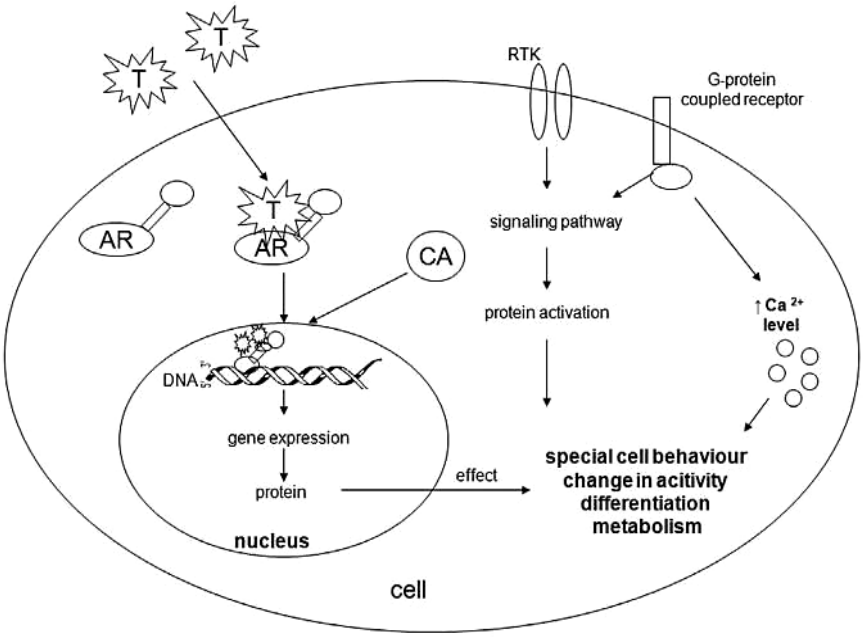

Figure 2. Genomic and non-genomic actions of testosterone (T). Unbound bioactive T interacts with the cytoplasm androgen receptor (AR). AR is also activated by dihydrotestosterone in a similar way. Ligand binding induces conformational changes of the receptor. T-AR complex forms dimers and acts as a functional transcription factor. Activated AR recognizes the androgen response element in the nucleus due its specific structure. Coactivators (CA) and RNA polymerase II are recruited for transcription initiation. Gene expression produces a pool of specific proteins that can affect cell characteristics, metabolism and activity. The non-genomic response is mediated via receptor-tyrosine-kinases (RTK) or G-protein coupled receptors. Subsequently, downstream signaling cascades are activated, that can result in genomic effect. Adapted from Durdiakova *et al.* (2011)(*35*).

Prenatal exposure to androgens has been shown to have an organizational effect on the brain circuitry involved in reproductive and metabolic regulation (*36*), leading to ovulatory dysfunction, infertility, increased risk of obesity, insulin resistance, and hyperlipidemia (*37*). These reproductive and metabolic dysfunctions are often associated with one of the most common female endocrine disorder, polycystic ovarian syndrome or in short, PCOS (Fig. 3). This multifaceted disorder affects more than 10 percent of reproductively aged women (*38*) and both genetic and environmental factors, such as exposure to excess T during a critical stage of prenatal development, are thought to play an important role in the manifestation of PCOS (*26, 39-40*). Exposure to androgens during the prenatal period may arise from either maternal or fetal hyperandrogenism. For example, circulating levels of testosterone are higher than normal during pregnancy in women with PCOS (*41*), proving a mechanism by which maternal androgen excess may disrupt normal development of the fetus. In addition, fetal hyperandrogenism can result from a hyperandrogenic fetal ovary (*42*), hyperandrogenic fetal adrenal cortex (*31*), or both. Furthermore, maternal hyperinsulinemia during pregnancy, a common occurrence in women with PCOS (*41*), induces excessive placental human chorionic gonadotropin (HCG) secretion leading to fetal ovarian hyperandrogenism (*42*). Therefore, both maternal and fetal hyperandrogenism can provide a mechanism by which the fetus is exposed to excess androgens during a critical period of development and may by permanently programmed to develop PCOS.

As the most frequent cause of anovulatory infertility (*43*), PCOS is characterized by a constellation of reproductive and metabolic deficits, including hyperandrogenemia,

polycystic ovaries, disrupted menstrual cycles and fertility complications, hypersecretion of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH), insulin resistance, and hyperinsulinemia (*44-48*). According to the 2003 Rotterdam consensus criteria, the presence of any two of three of oligo-ovulation or anovulation, hyperandrogenism (clinical or biochemical or both), and polycystic ovaries, fulfills a diagnosis of PCOS (*49*). In women with PCOS, the primary site associated with excess production of androgens are the ovaries (*50-51*). Although the etiology of excess ovarian androgen production is unknown, elevated levels of LH are believed to play a role in increasing production of androgens in the theca cells of the ovaries (*50*). These elevated LH levels may be due to LH hypersecretion by the pituitary gland or may be a result of increased amplitude and frequency of LH pulse. Nonetheless, this type of LH profile is believed to be a secondary reaction to an increase in GnRH from the hypothalamus (*45*). Indeed, women with PCOS have persistently rapid LH (GnRH) pulse frequency, reaching a frequency of one pulse per hour, without the normal cycle variation seen in ovulatory women (*52*). This rapid GnRH pulsatility results in preferential secretion of LH over FSH, and may help explain the elevated LH levels and increased LH to FSH ratios characteristic of PCOS (*53*).

Insulin resistance with compensatory increases in circulating insulin levels are also common features in PCOS, occurring in 30 % of lean and 75 % of obese women with PCOS (*54*). As such, insulin may exert its effects at the level of the hypothalamus, pituitary or ovaries, contributing to the elevated levels of LH. In vitro studies have shown

that insulin stimulates androgen synthesis in the thecal cells of the ovary by increasing LH-induced androgen production (*55*).

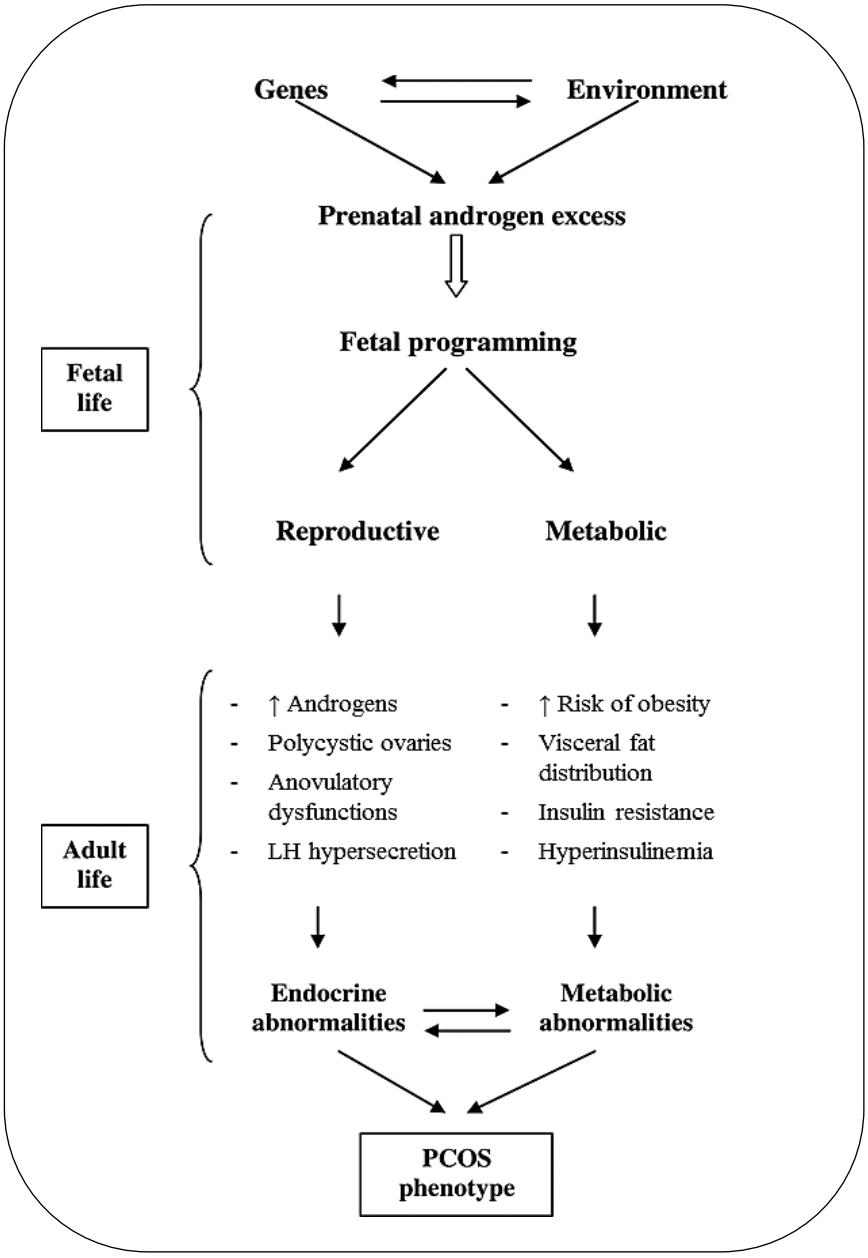

Figure 3. Prenatal Testosterone (T) excess and the development of polycystic ovarian syndrome (PCOS) phenotype in adulthood.

1.3 Insulin sensitizers and anti-androgens in the management of PCOS symptoms

Increased insulin resistance, hyperandrogenism and obesity have a significant impact on women's menstrual cyclicity and reproductive health. It is logical to assume that therapies promoting a decrease in circulating insulin or androgen levels will improve some undesirable symptoms of PCOS. Insulin sensitizers such as Rosiglitazone are widely used in the treatment of hyperinsulinemia in women with PCOS (*56*). Rosiglitazone works by binding to the peroxisome proliferator-activated receptors-γ (PPAR- γ) expressed in fat cells and subsequently increasing the cell's responsiveness to insulin (*57*). In a study by Yilmaz *et al.* (2005), Rosiglitazone treatment for 4 months reduced hyperinsulinemia and restored regular menstrual cyclicity in 60 percent of lean and 50 percent of obese PCOS patients (*58*). It is important to take into account weight loss as a significant contributor to the improvement of the metabolic and reproductive dysfunctions associated with PCOS (*59-60*). In a study by Pasquali *et al.* (1989), an average weight loss of 10-30 lbs. was shown to improve hyperinsulinemia and hyperandrogenism and to recover normal menstrual cyclicity in 30 percent of the patients studied (*61*). Considering that 50 percent of PCOS women are obese (*59*) and up to 70 percent are insulin resistant (*62*), weight loss should be an important part of any treatment regime in women with this syndrome.

Anti-androgens such as Flutamide have been used in women with PCOS mainly for the treatment of hirsuitism (*63-66*). Flutamide works by competitively inhibiting the binding of testosterone and its metabolite, dihydrotestosterone (DHT), to the androgen receptor

(*67*). In women with PCOS, Flutamide treatment significantly alleviates a majority of the clinical and biochemical androgenic manifestations; however it has a minimal effect on insulin sensitivity (*68-70*). As such, a combination of Flutamide with an insulin sensitizer may be more effective in improving a wider range of PCOS symptoms (*71*).

1.4 Developing animal models of PCOS

The development of reliable animal models of PCOS has proven very useful in understanding the pathogenesis of this syndrome, and particularly its reproductive and metabolic consequences. Since the 1960s, a variety of animal models, including rodents, sheep, and non-human primates, have been utilized to study the origins and pathology of PCOS (*22, 72-74*). Studies in both humans (*31*) and animals (*24, 40, 75-76*) have shown that prenatal exposure to excess T affects the development of the hypothalamus-pituitary-gonadal (HPG) axis and contributes to reproductive and metabolic dysfunction in adults. The majority of the human data documenting the permanent organization effects of prenatal T exposure are derived from women with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency. Females with classic 21-hydroxylase deficiency are exposed to excess androgen prenatally due to excessive adrenal androgen synthesis (*77*). These women also display PCOS-like phenotype, including hyperandrogenism, menstrual irregularities, and elevated LH levels (*77*). When LH secretion levels are compared in women with classic CAH and late-onset 21-hydroxylase deficiency, only women with the classic form, who have been exposed to androgens prenatally, show elevated levels of LH

and increased LH response to GnRH agonist (*31*). This suggests that exposure to androgens, whether derived from fetal adrenal or ovarian sources, or both, during a critical period of prenatal development, might provide a crucial hormonal insult that is necessary for the development of complex disorders such as CAH and PCOS in adulthood. Animal studies have provided further support for the effects of early exposure to androgens on neuroendocrine function. For example, female rhesus monkeys (*78-79*), sheep (*26, 80-81*) and rats (*82-83*) exposed to T during the prenatal period, develop abnormal gonadotropin secretion and elevated levels of LH, polycystic ovaries, and progressive disruptions in ovulatory cycles during adulthood.

1.5 Prenatal testosterone treated sheep model

In order to investigate the effects of prenatal T exposure on the brain, peripheral tissues and social behaviors, our research group has developed a prenatal T-treated sheep model of PCOS, which has been widely studied and documented (*37*) (Fig. 4). Therefore, along with my specific research aim to examine the effects of prenatal T on hypothalamic neurons, research contributions from our collaborators complement our findings and provide a "whole systems" approach of this disease model. Sheep are excellent models for studying developmental programming of adult disorders and have long been used to study fetal development (*84*). With a gestation length of 147 days, and time of puberty in females at 28 weeks, the sheep is ideally suited for integrative studies that investigate progression of reproductive ⁄ metabolic disruption from the initial prenatal insult to manifestation of adult consequences. The sheep's neuroendocrine aspects of reproductive

cyclicity are also comparable to those in the human (*85-86*). In addition, sheep are reliable subjects for assaying hormone levels as large quantities of blood can be sampled with ease and minimal stress to the animal.

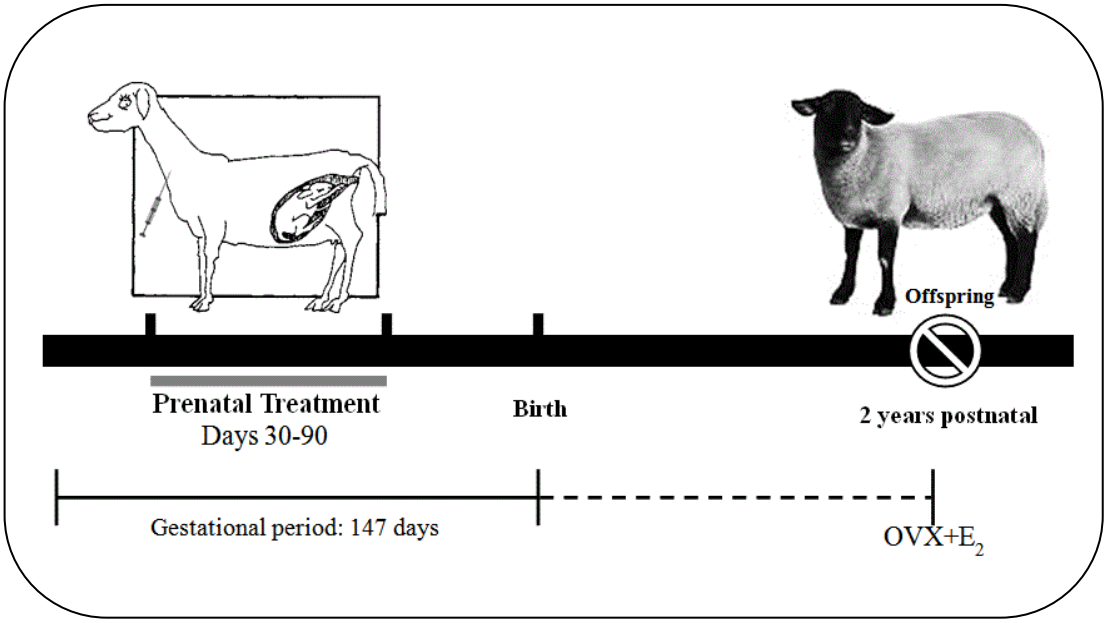

Figure 4. Schematic diagram illustrating the prenatal testosterone (T) sheep model. The pregnant mother is injected with T twice weekly from gestational days 30-90. Two years after birth, the adult prenatal T treated female offspring is ovariectomized (OVX), receives an estradiol implant (designed to mimic follicular phase levels of E_2), sacrificed, and the brain is collected for research.

1.6 Focusing on the hypothalamus

The main site of action for androgen induced reproductive dysfunctions appears to be at the level of the hypothalamus. Similar to the male, the hypothalamic–pituitary component of the reproductive axis in prenatal T treated females is unable to generate a GnRH-

induced LH surge in response to increasing levels of estradiol (E_2) (*87-89*). However, normal ovarian function appears secondary to and dependent on normal hypothalamic function. For example, removing the ovaries from non-cycling, perinatally androgenized female rats and transplanting them into normal females, reestablishes ovulatory function (*90*). Conversely, removing ovaries from normal females and transplanting them into perinatally androgenized females terminates ovulatory function (*90-91*). The role of the pituitary also appears to be dependent on normal hypothalamic function. For example, in the perinatally androgenized female rat, providing electrical stimulation to the hypothalamus is sufficient to induce ovulation (*92*). Although prenatal T treatment results in alterations at the level of the brain and periphery, these studies identify the hypothalamus as the major site for early life testosterone excess programming of anovulation.

1.7 Regulation of metabolic homeostasis by the AgRP and POMC neurons of the hypothalamus

In today's society, the prevalence of obesity is increasing worldwide, reflecting environmental factors such as over consumption and decreased physical activity. Maintaining energy balance requires establishment of equilibrium between energy intake and energy expenditure. The arcuate nucleus of the hypothalamus (ARC) is a central point of integration for various hormonal signals which regulate energy homeostasis. Within the ARC, the melanocortin system, comprising of proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) co-expressing neurons

(*93*) and agouti-related protein (AgRP)/neuropeptide Y (NPY) co-expressing neurons (*94-96*), is crucial for normal energy homeostasis. In the anorexigenic POMC neurons, the neuropeptide precursor POMC is cleaved to α-melanocyte stimulating hormone (α-MSH) (*97*), activates melanocortin 3 and 4 receptors (MC3R and MC4R) expressed on secondary neuron populations, located in the paraventricular nucleus (PVN) of the hypothalamus, among other nuclei (*98*). Thus, membrane depolarization of POMC neurons leads to α-MSH release, MC3/4R activation and ultimately decreases food intake and increases energy expenditure (*98*). The orexigenic AgRP acts by blocking α-MSH mediated activation of the MC3/4R, thus inhibiting α-MSH action and increasing appetite and energy expenditure (*99*). There is extensive evidence supporting the critical role of AgRP and POMC in the regulation of metabolic homeostasis (Fig. 5). For example, null POMC mice (-/-) exhibit hyperphagia, are obese and have a lower basal metabolic rate (*100*) and humans with POMC mutations are obese and hyperphagic (*101*). In rodents and sheep, central administration of AgRP increases food intake (*102-103*), while chronic exposure to exogenous AgRP causes continued hyperphagia resulting in obesity in rodents (*104*). Moreover, sheep fed an ad libitum diet have decreased AgRP neuronal numbers (*103, 105*) while those fasted have increased AgRP mRNA expression and more AgRP-immunolabelled neurons (*103, 106*).

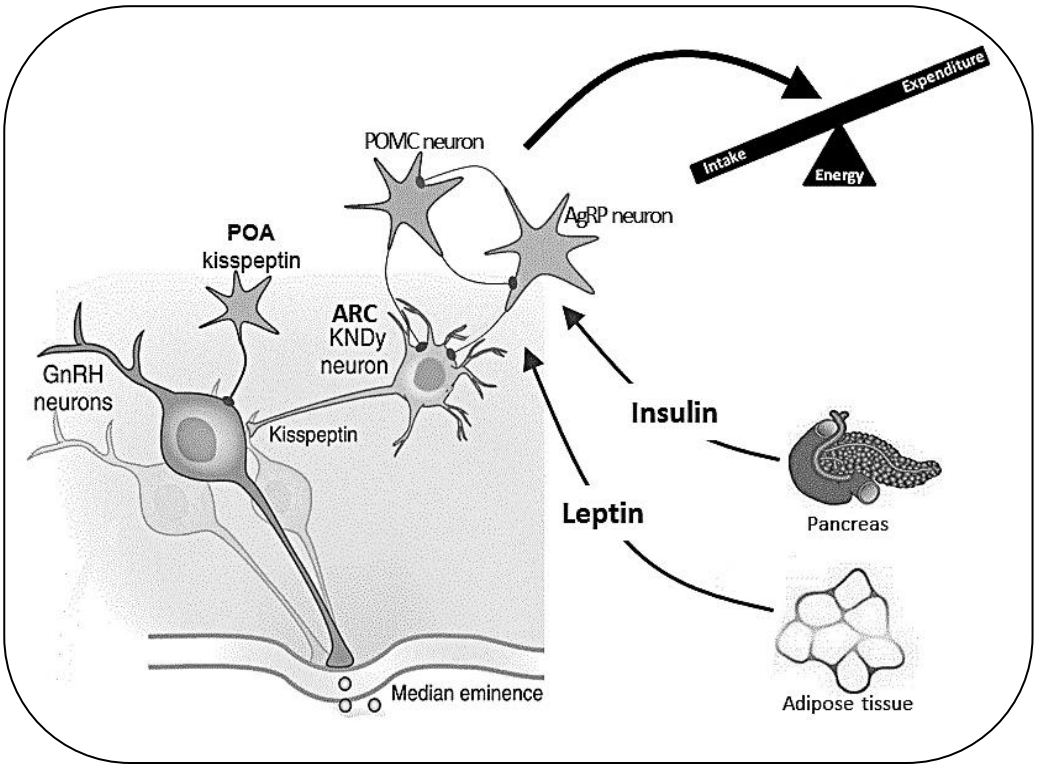

Figure 5. Schematic diagram illustrating the interactions between the metabolic and reproductive neurons of the arcuate nucleus of the hypothalamus (ARC) and their effects on energy homeostasis. *Abbreviations*: ARC, arcuate nucleus; POA, preoptic area; AgRP, agouti-related peptide; POMC, proopiomelanocortin; KNDy, kisspeptin, neurokinin B, dynorphin; GnRH, gonadotropin releasing hormone.

AgRP and POMC neurons also express nuclear androgen receptors (AR) (*107*), indicating a possible site of action for androgenic regulation of the metabolic neuronal circuitry as well as a potential for disturbance by excess prenatal testosterone (T) exposure. Our lab has shown that prenatal T treated ewes have a significantly higher number of AgRP-expressing neurons in the ARC, but no change in POMC neurons compared to controls (*107*). This finding reveals that prenatal T exposure differentially

alters the metabolic neurons of the ARC and also provides a framework for ongoing metabolic studies with the prenatal T sheep model.

1.8 Overview of reproductive circuitry (Kisspeptin/KNDy and GnRH neurons) and effects of prenatal T treatment

In sheep, kisspeptin neurons are located in the hypothalamus, with two distinct populations-one in the preoptic area (POA) and the other in the arcuate nucleus (ARC) (*108-109*). In the ARC, but not in the POA (*110*), kisspeptin is colocalized with two other neuropeptides: the opioid peptide, dynorphin and the tachykinin peptide, neurokinin B (NKB). The colocalization of these three peptides is nearly 100% in the ARC, and thus this subpopulation has been collectively termed the "KNDy" (kisspeptin, neurokinin B, dynorphin) neurons (*110*). ARC KNDy neurons also show a high degree of colocalization with the gonadal hormone steroid receptors estrogen receptor alpha (ERα, >90%) (*111*), progesterone receptor (PR, >80%) (*112*), and androgen receptor (AR, >60%) (*113*). The colocalization of steroid receptors with kisspeptin has also been shown in the POA, but to a lesser extent (ERα> 50% and AR>25%) (*111, 113*). Our lab has recently shown that both populations of kisspeptin neurons (ARC and POA) are activated during the GnRH surge, but only ARC kisspeptin (KNDy) neurons are activating during pulsatile secretion of GnRH (*114*). In support of this, our lab has also shown that GnRH neurons receive inputs form POA kisspeptin neurons as well as ARC KNDy neurons (*115*) (Fig. 6). Furthermore, GnRH is directly stimulated by kisspeptin, and GnRH neurons express the kisspeptin receptor (*116-117*). In terms of coordinating GnRH pulsatile activity, the

KNDy neuropeptides are believed to collectively to modulate GnRH release, each playing distinct a role in conveying the feedback signals of estrogen and progesterone onto GnRH neurons (*118*). As a network, the ARC KNDy neurons are also reciprocally connected and thus may play a role in the synchronous activity of the ARC, an aspect which is associated with the pulsatile release of GnRH. Based on this and other features, the KNDy population has been proposed to be a critical component of the "GnRH pulse generator" with the three peptides serving roles as the start signal (NKB), stop signal (dynorphin) and output (kisspeptin) responsible for each pulse (*110, 115, 118-121*).

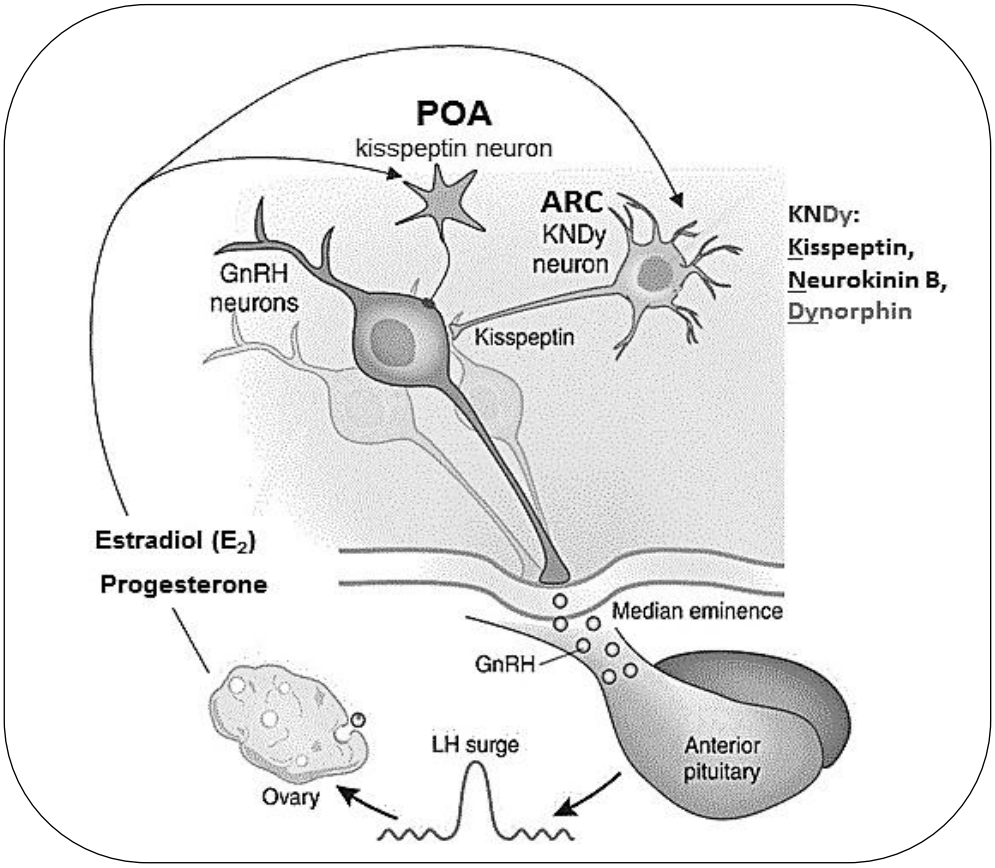

Figure 6. Schematic diagram illustrating the interactions between the arcuate (ARC) and preoptic area (POA) kisspeptin neurons with gonadotropin releasing hormone (GnRH) neurons and their effects on the hypothalamo-pituitary-gonadal (HPG) axis and reproductive function.

Previously, our lab showed that prenatal T treatment reduces the number of dynorphin, neurokinin B (NKB), and progesterone receptor-ir neurons in the ewe ARC, however the number of kisspeptin neurons remains high and at levels similar to control females (*122*). Thus, there is an imbalance between inhibitory (dynorphin) and stimulatory (kisspeptin)

neuropeptides in this population, and that this imbalance may play a role in the disrupted steroid feedback that is typical of the prenatal T model. Throughout my master's studies I continued to explore the effects of prenatal T treatment on the KNDy-GnRH circuitry and showed that prenatal T treatment decreased the reciprocal connections between KNDy neurons and their contacts to GnRH neurons. This reduced connectivity may alter the synchronicity of the KNDy neurons and result in a dilution of the signal to the GnRH neurons. This could partially explain the delayed and dampened GnRH/LH surge seen in prenatal T treated females and possibly the absence of ovulatory cycles seen in women with PCOS. During my master's studies I also found that the KNDy neurons of prenatal T females had an increased soma size. This change was restricted to the ARC KNDy neurons, as no soma size differences were found in POA kisspeptin, AgRP or POMC neurons. This selective hypertrophy is also seen in the hypothalamus of postmenopausal women (*123*) and is believed to be a secondary response to ovarian failure (*124-125*) and associated with declining levels of circulating E_2 concentrations (*126*). Overall, prenatal T treatment results in morphological changes both in the synaptology of the KNDy population and its connections to GnRH, as well as its neuronal size. These changes may contribute to defects in the ability of the KNDy population to convey steroid feedback signals to GnRH neurons.

While the KNDy neuronal population is a target for the organizational effects of prenatal T (*127*), the specific mechanisms by which this programming occurs are still under investigation.

1.9 Leptin and insulin as regulators of metabolic activity

Metabolism and energy homeostasis are regulated by the hormones leptin and insulin, which circulate at levels proportional to body fat stores (*128-129*). Levels of these circulating hormones serve as an indicator of energy stores to the brain and act to increase or suppress food intake and energy expenditure (*130*). The appetite stimulatory, AgRP, and inhibitory, POMC neurons of the ARC are key targets of leptin and insulin as they both express leptin and insulin receptors (*131-134*) (Figure 5). Normally, increases in leptin and insulin are associated with increased POMC expression (*98, 135*) and with decreased AgRP/NPY expression, which ultimately decreases food intake (*98, 135*).

1.10 Insulin signalling in the brain

Insulin is a metabolic hormone involved in the homeostatic control of energy balance (*136*). Insulin acts both acutely - to inhibit food intake, and chronically - as it circulates in proportion to fat stores to regulate body weight (*137*). When a meal is consumed, blood glucose is increased and insulin is rapidly secreted from beta (β) cells in the islets of Langerhans in the pancreas to promote glucose uptake from the blood (*129*). Accordingly, insulin secretion is increased during food intake and increased energy states and decreased during periods of food restriction.

Insulin acts both peripherally and centrally. To enter the brain, insulin crosses the blood-brain barrier (BBB), in levels proportional to circulating insulin, by a saturable receptor-mediated transport mechanism (*138-140*). Insulin can also access the brain by circumventricular regions which lack the BBB (*141-142*). The concentration of insulin in

the brain is influenced by diet in both animals and humans. Eating increases insulin in the hypothalamus (*143*), while fasting decreases brain insulin levels (*144-145*). Central administration of insulin causes a reduction in food intake in rodents (*146*), sheep (*147*), and nonhuman primates (*148*) and ultimately results in decreased body weight (*149*).

Insulin receptors (IR) are expressed in neurons and glia (*145*) and distributed throughout the brain (*150-152*). The highest proportions of IRs are found in the hypothalamus, particularly in the arcuate nucleus (ARC) (*153*); however the cerebral cortex, cerebellum, hippocampus, and olfactory bulb also contain high levels of IR (*154*). Insulin receptor mRNA is found in the somata of the neurons, whereas protein is present in cell bodies and axon terminals (*145, 155*). In rodents, various populations of neurons in the brain that are involved in energy homeostasis express insulin receptors, including ARC AgRP (*156*) and POMC neurons (*133*). As previously mentioned, insulin acts to increase POMC gene expression (*133*) and decrease AgRP release (*157*). The insulin receptor signalling pathway consists of an extracellular alpha subunit that binds the insulin and an intracellular beta subunit that relays the signal to the cell (*158-159*). Binding of insulin induces a conformational change in the receptor inducing autophosphorylation of the b-subunit, subsequently activating intrinsic tyrosine kinase activity of the receptor. Insulin receptor substrate (IRS) proteins are then recruited to the plasma membrane, which become phosphorylated on tyrosine residues, subsequently recruiting additional signalling proteins regulated by various protein kinases (*160*). The two major kinases involved are phosphoinositide-3kinase (PI3K) and the mitogen-activated protein kinase (MAPK) (*161-162*). Biological responses are then initiated through phosphorylation and

activation of these major cellular signal transduction cascades, which regulates gene transcription implicated in growth and metabolism as depicted in Fig. 7 (*163-165*).

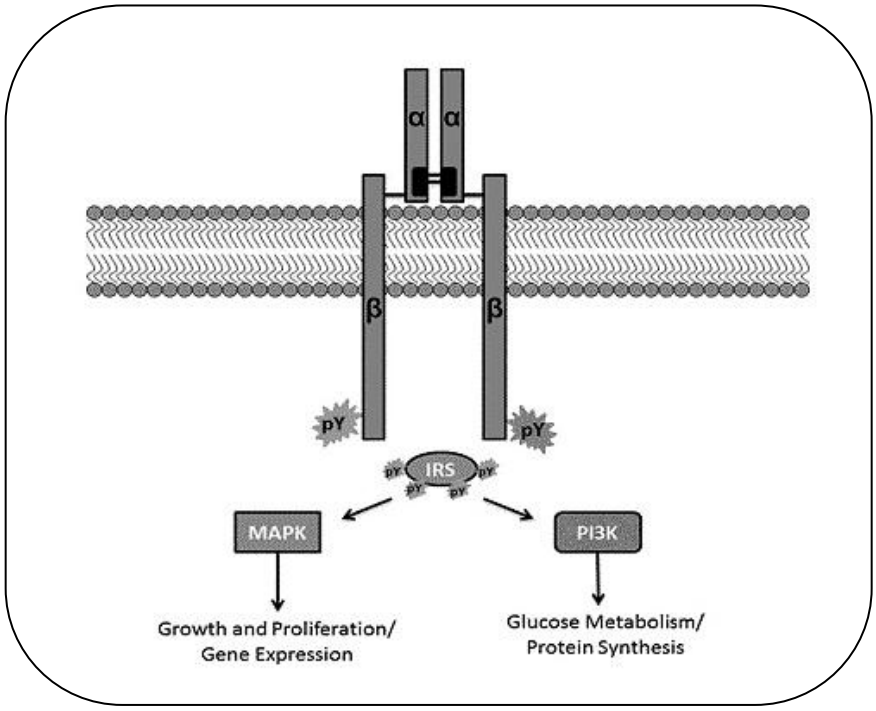

Figure 7. Insulin receptor signaling pathways: binding of insulin to the α-subunit of the insulin receptor activates tyrosine autophosphorylation of the β-subunit and subsequent downstream signalling pathways. *Abbreviations:* pY, phospho-tyrosine; IRS, insulin receptor substrates; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase. Adapted from Chiu and Cline (2010) (*165*).

1.11 Insulin Resistance

The strong link between insulin resistance, subsequent hyperinsulinemia and obesity has been recognized for more than 50 years (*166-167*). Insulin resistance is a physiological condition in which the body's cells become resistant or insensitive to the actions insulin

(via alterations in insulin receptors). When tissues do not appropriately respond to physiologic concentrations of insulin, higher concentrations of insulin are required to maintain normal blood glucose levels. This characterizes insulin resistance and hyperinsulinemia (*168-170*).

Insulin resistance is one of the most common symptoms of PCOS, as marked by impaired glucose transported into skeletal tissue (*171-173*). It is estimated that about 50-70% of all women with PCOS have some degree of insulin resistance (*174-175*). Treatment with insulin-sensitizing drugs improves insulin resistance as well as ovulatory function and fertility (*58, 176*), even without associated weight loss (*177*). Thus, it is believed that insulin resistance is an important component in the development of PCOS and intensity of the phenotype. Insulin sensitizers have also been show to also reduce androgen levels and alleviate symptoms associated with hyperandrogenemia (*178*). As such, insulin resistance and subsequent hyperinsulinemia are now recognized as indicators of hyperandrogenism in the majority of PCOS women (*48, 179*). In support of this, hyperinsulinemia, together with elevated LH levels, has been shown to exacerbate hyperandrogenemia by increasing androgen production in theca cells of the ovary (*180-182*). This activates the enzyme P450c17α in the ovary, which is critical in the biosynthesis of androgens (*183*). Moreover, insulin suppresses sex hormone binding globulin (SHBG) produced by the liver, permitting an increase in unbound plasma testosterone (*184*). Obesity and increased visceral fat distribution (which has been implicated in the development of insulin resistance (*185-186*)) may also contribute to site specific androgen excess (*187*). Human adipose tissue is capable of synthesizing

androgens via the enzyme 17β-hydroxy steroid dehydrogenase (17β-HSD), which can catalyzes the conversion of Androstenedione (A) to T (*188*). In this way, increased visceral fat, insulin resistance and hyperandrogenemia form a self-perpetuating cycle, further intensifying the PCOS phenotype. Indeed, more than 50% of women with PCOS display both insulin resistance and abdominal obesity (*189*).

1.12 Leptin signalling in the brain

Leptin is a protein hormone encoded by the *ob* gene that is expressed primarily in adipose tissue, but also in the stomach, placenta and mammary gland (*190*). Circulating leptin levels are proportional to fat stores (*135*, *191*) and therefore, higher leptin levels are observed in obese individuals (*192*). In the brain, leptin also serves as an indicator of peripheral energy stores and signals suppression of food intake and increased energy expenditure (*193*). Leptin operates in a regulatory feedback loop to maintain body weight and metabolic homeostasis (*194*). During states of weight gain and increased fat depots, leptin secretion is increased from adipose tissue and acts on leptin receptors in the brain to signal a decrease food intake and increasing energy output in attempt to lose weight and maintain metabolic homeostasis. In states of weight loss, leptin secretion is decreased permitting increased food intake and decreased energy expenditure to promote weight gain and achieve metabolic homeostasis.

Leptin mediates its effect through specific leptin receptor isoforms, which are widely distributed in many tissues (*190, 195*). The short form of the leptin receptor (LepRa) is expressed in the choroid plexus and transports leptin into the CSF by a saturable transport

system past the BBB (*195*), however leptin can also enter the brain through circumventricular organs that lack BBB. The long form of the leptin receptor (LepRb) is expressed abundantly in the hypothalamus and is involved in metabolic regulation (*135*, *196*).

Binding of leptin to the extracellular domain of its receptors mediates the activation of the intracellular associated Janus Kinase (JAK) resulting in JAK2 autophosphorylation on tyrosine residues (*193*). This is a critical step in leptin signalling as LepRb does not have any enzymatic activity of its own (*197*). Activated JAK stimulates the phosphorylation of residues on the intracellular domain of the receptor Tyr985, Tyr1077, and Tyr1138. When phosphorylated, each of these residues recruits downstream signalling proteins. Signal transducer and activator of transcription (STAT) 3 is recruited to the phosphorylated Tyr1138 residue (Fig. 8). This recruitment subsequently mediates phosphorylation and activation of STAT3. When activated, STAT3 translocates to the nucleus and activates transcription of target genes (*198*).

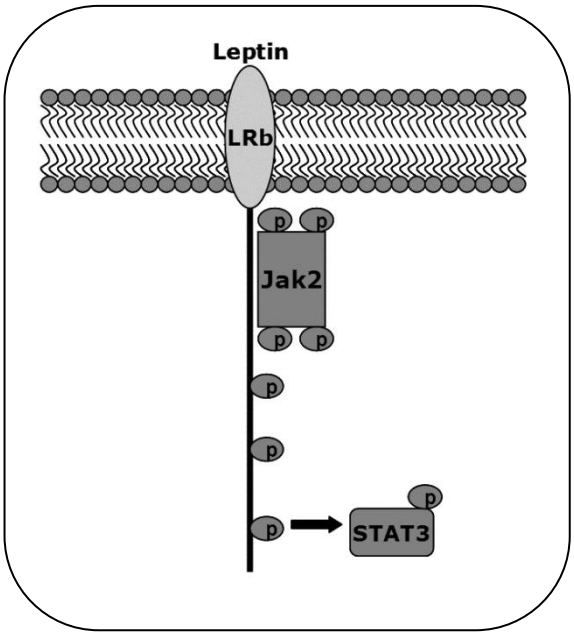

Figure 8. Long form of the leptin receptor and signalling pathway. Binding of leptin to the extracellular domain of the long form of the leptin receptor mediates phosphorylation of the intracellular domain of the receptor and results in transcription of target genes. *Abbreviations:* LRb: leptin receptor b; p: phospho-tyrosine; Jak2: Janus Kinase 2, STAT3: signal transducer and activator of transcription 3. Adapted from Villanueva and Myers (2008) (*199*).

1.13 Link between metabolism and reproduction

As previously discussed, reproductive function is regulated by the complex interaction of the hypothalamus, pituitary and gonads, or the HPG axis. Proper function of the HPG axis is gated by metabolic and nutritional factors. Hence, fertility is dependent on nutrition and stored energy availability. If energy reserves are insufficient, the reproductive axis is suppressed and appetite is stimulated to compensate. Caloric restriction not only decreases insulin secretion, it also reduces levels of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (*200*). Our knowledge of the neurohormonal pathways that are responsible for the metabolic

control of gonadotropic function has increased substantially since the early 1990's. Among the many endocrine regulators involved in the control of reproduction and metabolism, the hormones leptin (*201-204*) and insulin (*205-206*) play an essential role in communicating energy homeostasis to the reproductive axis and modulation of GnRH secretion.

For example, female mice with neuron-specific knockout of the insulin receptor (NIRKO) exhibit obesity, reduced LH secretion and impaired ovarian follicle maturation (*205*). However, it has not yet been shown *in vivo* that (GnRH) neurons express insulin receptor (IR), suggesting that the influence of insulin on GnRH secretion and reproductive function may involve afferent insulin sensitive neurons. One of the first links between leptin and reproduction came from the observation that mice with a mutation in the ob gene (*ob/ob mice*) are infertile (*207*). These mice are also obese, hyperphagic and have a lowered metabolic rate (*208-209*). Correcting leptin deficiency in these mice by leptin administration, restores fertility (*210*).

Leptin activates the neuroendocrine reproductive axis in many species including rodents (*210-212*) and primates (*213*) by stimulating GnRH and LH secretion. However, various studies have shown that few, if any GnRH neurons expressing leptin receptors (*132, 213*). Therefore, leptin's influence on GnRH secretion and reproductive function may likely involve and indirect mechanism of communication with GnRH, possibly via leptin sensitive interneurons upstream of GnRH-releasing neurons (*214*).

It is tempting to hypothesize that the ARC populations comprising of KNDy, AgRP and POMC neurons and the POA kisspeptin neurons may be targets of leptin and insulin

signaling. As previously discussed, both leptin and insulin mediate AgRP and POMC activity and these neurons have been shown to play a role in regulating GnRH secretion. In sheep, NPY fibers (co-expressed with AgRP (*94*)) directly contact GnRH neurons (*215*) and this interaction has been shown to have an inhibitory effect on LH secretion (*216*). In fact, chronically elevated levels of NPY can block the GnRH/LH preovulatory surge (*216*). The ARC POMC neurons have also been shown to make direct connections to GnRH neurons and regulate GnRH activity in the mouse (*217*) and hamster (*218*). However, unlike NPY, the melanocortins (post-translational products of POMC) stimulate reproductive function (*219*).

Nutritional status can also influence kisspeptin expression. In rodents, fasting decreases kisspeptin mRNA (*220-221*), while leptin administration increases kisspeptin mRNA (*222*). However, there have been discrepancies in determining how leptin acts to stimulate GnRH, whether through a direct or indirect mechanism acting on kisspeptin neurons. The extent of LepRb expression with kisspeptin neurons has been a topic of debate for some time. In the mouse, it has been reported that the AVPV kisspeptin neurons do not coexpress LepRb, but that up to 40% of ARC kisspeptin neurons coexpress LepRb (*223*). Our research group has previously shown that in mice and sheep, leptin treatment does not directly activate ARC or AVPV (POA) kisspeptin neurons or GnRH neurons, via induction of pSTAT3 (*224*). As such, leptin signals influencing reproductive function may be relayed to the kisspeptin and/or GnRH neurons indirectly, via upstream leptin-sensitive neuronal populations.

The direct modulation of GnRH activity by insulin signaling has been highly debated as a direct link between insulin and GnRH activity has only been shown in vitro, in an immortalized GnRH cell line (*225*). As such, kisspeptin has been proposed as one of the key candidates in relaying insulin signals to the GnRH neurons. A recent study in mice showed that more than 20% of ARC kisspeptin neurons colocalized IR (*226*). Although, deletion of IR specifically from these neurons does not affect fertility; it does delay the onset of puberty. Moreover, male and female mice with GnRH specific IR deletions also display normal pubertal timing and fertility (*227*). These studies suggest that IR expression in kisspeptin or GnRH neurons do not play a critical role in the maintenance of reproductive function in mice. However, in a separate animal such as the sheep, the role of insulin signaling in these neurons has not been explored. A decrease in IR with kisspeptin or GnRH may very well interfere with reproductive as well as metabolic function, especially in a disease model where these basic functions have been programmed in an abnormal manner.

Nevertheless, prenatal T-treated sheep show metabolic and reproductive dysfunctions involving complex systems that are highly interlinked. Disruptions in the organization and development of these systems can exacerbate reproductive and metabolic dysfunctions during adulthood.

1.14 My Research Goals:

There is a remarkably close link between the homeostatic metabolic and reproductive neuroendocrine systems. Female animals show dramatic decreases in the rates of reproduction during periods of continual negative energy balance (*228-231*), suggesting that the state of energy balance has an important role in determining when and if it is appropriate for an animal to take on the energetically expensive demands of reproducing. Alterations of energy balance are associated with clear changes in activity of the reproductive axis. During negative energy balance, there is suppression of pulsatile GnRH/LH secretion (*229-230, 232*) and both leptin (*233-234*) and insulin (*235-236*) can affect GnRH/LH pulse frequency. Thus, it may be that some or all of the reproductive defects as a result of prenatal T are in fact due to metabolic dysfunction at the level of the brain or periphery. Therefore, to further understand how prenatal T organizes the female brain, I will be investigating the effects of prenatal T on the reproductive as well as metabolic neural circuitry of the brain, focusing on two critical peptide hormones: insulin and leptin.

My dissertation work is divided into four main parts.

1) Prenatal T treated females are noticeably masculinized and display male-type behaviors; however we cannot accurately measure circulating androgen levels in these females. Therefore, the first objective aims to show that prenatal T ewes display functional hyperandrogenemia by characterizing AR-immunoreactivity (ir) in areas of the brain controlling reproduction and metabolism (ARC and POA) as well as areas controlling sexual behaviours behaviors (Amygdala and Basal nucleus of the stria

terminalis), with the expectation that AR-ir will be sexually dimorphic and prenatal T-treated ewes will have increased AR-ir compared to control females. Furthermore, because prenatal T treated females show distinct alterations in the AgRP and KNDy populations, this aim will also examine the effects of prenatal T treatment on AR colocalization in these populations.

2) Insulin plays an important role in maintaining energy balance relaying energy availability signals to the reproductive axis. However, prenatal T treated sheep show impaired insulin signalling displayed as insulin resistance and hyperinsulinemia. As such, the second objective aims to i) determine if ARC KNDy, AgRP and POMC neurons, POA kisspeptin neurons and GnRH neurons express insulin receptor (IR) and ii) the effects of prenatal T treatment on the degree of IR colocalization with these populations.
3) Hyperandrogenemia and hyperinsulinemia have been shown to play a significant role in the programming and maintenance of the symptoms associated with PCOS as well as prenatal T treatment. As such, the third objective aims to examine if prenatal and postnatal interventions with the androgen receptor blocker, Flutamide (F) and the insulin sensitizer, Rosiglitazone (R) are able to reverse the effect of prenatal T on the colocalization of AR and IR with the reproductive and metabolic neurons of the arcuate, the KNDy and AgRP neurons, respectively.
4) Leptin is an important metabolic signal regulating reproductive function; however the hypothalamic pathways through which leptin relays its influence on GnRH secretion remain to be defined. The fourth objective aims to characterize the hypothalamic

neuronal populations directly and indirectly targeted by leptin within the AgRP, POMC, KNDy and GnRH neuronal populations in order to elucidate a possible mechanism through which leptin can influence GnRH secretion.

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Chapter 2: Excess Prenatal Testosterone Increases Androgen Receptor Immunoreactivity in Hypothalamic Areas of the Adult Female Sheep Brain

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2.1 Abstract

Sex steroids play a key role in the development and function of the brain. Therefore, inappropriate exposure to excess steroid hormones is a major concern, especially in the female. In the brain, changes in steroid receptor expression can alter the pathways and mechanisms which regulate behavioral and physiological functions. Using prenatal testosterone (T), dihydrotestosterone (DHT) and estrogen (E)-treated female sheep as model systems, we tested the hypothesis that prenatal T excess alters androgen receptor (AR)-immunoreactivity (ir) in several areas of the brain including: the arcuate nucleus (ARC), the preoptic area (POA), the ventromedial hypothalamus (VMH), the amygdala (Am), and the bed nucleus of the stria terminalis (BNST) and compared our findings with rams and castrated (CAST) rams. We also analyzed the effects of prenatal T on AR-ir in kisspeptin and agouti-related peptide (AgRP) neurons as these populations play a key role in conveying reproductive and metabolic signals in the brain, respectively. Pregnant Suffolk ewes were injected twice weekly with T propionate, DHT propionate (a non-aromatizable androgen) or E in cottonseed oil, from days 30 to 90 of gestation. Changes in AR were analyzed at adult age (21 months) using immunohistochemistry. Control rams showed the highest AR-ir in all brain areas analyzed. Prenatal T and DHT, but not E, treatment resulted in an increase in AR-ir in the ARC and POA compared to control ewes and CAST rams. AR expression in kisspeptin and AgRP neurons was also increased in prenatal T females. Prenatal T and DHT treatment induced changes in AR-ir in the hypothalamus and specifically within kisspeptin and AgRP neurons and co-treatment

with the androgen blocker, flutamide reversed this effect, suggesting that prenatal organization of AR distribution and expression is organized by the androgenic actions of testosterone. Furthermore, its effects are restricted to hypothalamic regions and do not extend to associated limbic area such as Am and BNST. These findings provide us with a characterization of AR-ir in the brain as a consequence of different prenatal steroidal environments. In turn, this information will allow us to focus on the specific mechanisms targeted by prenatal T exposure that lead to altered behavioral and endocrine phenotypes seen in prenatal T treated female sheep.

2.2 Introduction

Sex steroids play a key role in the development and function of the brain. Therefore, inappropriate exposure to excess steroid hormones is a major concern, especially in the female. It has been previously shown that exposure to excess T during a critical period of prenatal development results in phenotypic, behavioral and reproductive abnormalities in humans (*1-3*) and other species such as monkey (*4-5*), sheep (*6-9*), rat (*10*), mouse (*11*), and guinea pig (*12*). For example, ewes prenatally treated with testosterone (T) from days 30-90 of gestation (T60) display multiple neuroendocrine and ovarian disruptions such as LH hypersecretion, disrupted estrogen negative feedback, disrupted positive feedback (manifested as a delayed and dampened LH surge) (*7*), and metabolic disruptions such as hyperinsulinemia and insulin resistance (*13*). In addition to reproductive and metabolic disturbances, these females also display altered sexual behavior presented as high numbers of male-typical mating behaviors directed at estrous females and aggression towards males (*14*). In the brain, changes in steroid receptor expression can alter the pathways and mechanisms which regulate behavioral and physiological functions. Thus, is it important to determine if prenatal T-treated ewes are hyperandrogenic during adulthood, as prenatal alterations in neuroendocrine organization may alter reproductive, metabolic and sexual behaviours by rendering these females more sensitive to the actions of androgens. As such, we focused on the main reproductive and metabolic centers of the brain, the arcuate nucleus (ARC) and preoptic area (POA), as

well as areas associated with sexual behavior, the ventromedial hypothalamus (VMH), amygdala (MeA) and bed nucleus of the stria terminalis (BnST), to determine whether these areas contain AR positive neurons and whether prenatal T treatment alters AR-immunoreactivity (ir). Furthermore, we analyzed AR-ir in two neuronal populations that have been shown to be critical in reproductive and metabolic function: ARC and POA kisspeptin neurons and the appetite stimulating agouti-related peptide (AgRP) neurons. The kisspeptin neurons (also co-expressing the neuropeptides neurokinin B and dynorphin in the ARC and known as KNDy neurons) have been shown to play a critical role in conveying steroid hormone information to GnRH neurons and thus play a critical role in the development and maintenance of normal reproductive behavior and fertility (*15-17*). In fact, kisspeptin is essential for the full preovulatory LH surge (*18*) (*19*), a mechanism that is impaired in prenatal T treated females (*6*). Furthermore, prenatal T treatment alters the balance of the KNDy neuropeptides resulting in a reduction in the expression of dynorphin and neurokinin B (NKB), but not kisspeptin within these neurons (*20*). Moreover, the KNDy neurons show a high degree of colocalization with the AR (*21*), suggesting that this population is a target for the programming effects of T. Along with reproductive dysfunctions, prenatally T-treated sheep are also metabolically compromised and show reduced insulin sensitivity and subsequent hyperinsulinemia (*13, 22*). Previously, our lab showed that prenatal T treatment significantly increases the number of AgRP neurons in adult females and these neurons also colocalize AR (*23*). As such, prenatal T, acting via AR during a critical period of brain organization may lay the framework for various neuroendocrine and behavioural alterations during adulthood. In

this study, we use prenatal T treated sheep as model systems, testing the hypothesis that prenatal T excess alters AR-ir in the ARC, POA, VMH, Am, and BnST, as well as with ARC and POA kisspeptin neurons and AgRP neurons.

2.3 Materials and Methods

2.3.1 Animals

Two experiments were included. Experiment 1 included control females (n=6), prenatal T females (n=6), prenatal dihydrotestosterone (DHT) females (n=4), prenatal estradiol (E) females (n=4), control males (n=5), and castrated males (n=4). To generate prenatal T-treated ewes, pregnant Suffolk ewes were administered twice weekly i.m. injections of testosterone propionate (100 mg/injection, catalog item T1875; Sigma- Aldrich, St. Louis, MO) suspended in cottonseed oil (catalog item C7767; Sigma-Aldrich) during gestational days 30–90 (term = 147 d), as previously described (*20*, *22-26*). This dose of testosterone propionate results in levels of T in the female fetus comparable to those seen in fetal males (*27*). Females treated prenatally with dihydrotestosterone (DHT) received two weekly 2 ml intramuscular injections of 100 mg dihydrotestosterone propionate (Steraloids, Inc., Newport, RI, USA) in cottonseed oil and females treated prenatally with estradiol (E) were implanted with one 30-mm Silastic implant (i.d. 0.22 cm; o.d. 046 cm; sealed with Silastic adhesive Type A, Dow-Corning corp., midland, MI) containing crystalline 17ß-estradiol (Sigma, St. Louis, MO) between days 30-90 of gestation. Control ewes received an equal volume of vehicle (2 ml cottonseed oil). All lambs were born in March and April. After weaning, they were maintained outdoors under natural photoperiods with a daily maintenance feeding and free access to water at the Sheep Research Facility of the University of Michigan (Ann Arbor, MI) until the age of 2 yr. Three-four weeks prior to tissue collection, endogenous steroid levels were normalized by bilateral ovariectomy and 17β-estradiol implants were inserted to mimic late follicular

phase of the estrous cycle as previously described (*28*). Control males and Castrated males were maintained under similar housing conditions.

Experiment 2 included four groups of females: control (n=5) and prenatal T-treated females (n=5) (as described above), females prenatally co-treated with T and androgen receptor antagonist flutamide (15 mg/kg; Sigma-Aldrich) (n=5), or females treated with flutamide alone (n=5) during gestational days 30-90. Flutamide was dissolved in dimethylsulfoxide (400 mg / ml) and injected s.c. daily as previously described (*29*). Lambs were born in March and April and were maintained outdoors under natural photoperiods with a daily feeding schedule and free access to water until the age of 2 yr. Three-four weeks prior to tissue collection, endogenous steroid levels were normalized by bilateral ovariectomy and 17β-estradiol implants were inserted to mimic late follicular phase of the estrous cycle as previously described (*28*).

2.3.2 Tissue collection

Animals received two i.v. injections of heparin (25,000 U given 10 min apart; catalog item 402588B; Abraxiz Pharmaceutical Products, Schumberry, IL), were deeply anesthetized with sodium pentobarbital (390 mg/ml/kg; Fatal Plus, Vortech, Dearborn, MI) and were decapitated. The heads were immediately perfused via both internal carotids with 6 liters of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3) containing 0.1% sodium nitrate (Sigma- Aldrich). The brains were removed, placed in 4% paraformaldehyde in 0.1 M PB overnight for post fixation at 4° C, and transferred into 30% sucrose in 0.1 M PB for cryoprotection. Frozen coronal sections through tissue

blocks containing preoptic area, septal nuclei, hypothalamus, or temporal lobe were cut (45 μm) using a freezing microtome (Microm HM400R; Walldorf, Germany) in 12 parallel series and stored at -20^{o}C in a cryopreservative solution (*30*) until being processed.

2.3.3 Immunohistochemistry for AR, AR/kiss or AR/AgRP

Sections were processed free floating with gentle agitation and all steps were performed at room temperature. Antibodies were dissolved in incubation solution consisting of 0.1M PBS, 0.4% Triton X-100 (catalog item BP151- 500; Sigma-Aldrich) containing 4% normal goat serum (NGS) (catalog item 005-000-121; Jackson Immuno Research Laboratories, West Grove, PA) and sections were washed extensively with 0.1 M PBS (pH 7.35) between steps. Prior to incubation with first primary antibody sections were incubated with 1% hydrogen peroxide (10 min, H_2O_2; catalog item H325; Fisher Scientific, Pittsburgh, PA) and incubation solution (1hr) to prevent non-specific background labeling. Within each experiment, tissue sections from all experimental groups were processed simultaneously.

One series of tissue sections was incubated with rabbit anti-AR (1: 200 dilution, 17 h; Santa Cruz, sc-816, lot F1711), biotinylated goat anti-rabbit IgG (1:500, 1 hr; catalog item BA-9200; Vector Laboratories, Burlingame, CA), avidin-biotin horseradish peroxidase complex (ABC ; 1:500, 1 hr, catalog item PK-6100; Vector Laboratories), and 0.02% 3, 3’-diaminobenzidine tetrahydrochloride (DAB, Sigma-Alderich) with 0.08%

nickel sulfate (10 min; diluted in PB containing 0.012% H_2O_2). One series of tissue sections was first immunoprocessed for AR and subsequently incubated with rabbit anti-kisspeptin (1:300,000 dilution, 17 h; kp10, lot 564; gift from Dr. Alain Caraty, Nouzilly, France) or guinea pig anti-AgRP (1:1000 dilution, 17 h, C-GPAAGRP, Lot AS-506, Antibodies Australia), biotinylated goat anti-rabbit IgG (1:500, 1 hr; catalog item BA-9200; Vector Laboratories, Burlingame, CA), ABC (1:500, 1 hr, catalog item PK-6100; Vector Laboratories), and 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Alderich,10 min; diluted in PB containing 0.012% H_2O_2). Tissue sections were mounted onto Superfrost/Plus Microscope Slides (Fisher Scientific), dehydrated with alcohol and cover-slipped with Depex Mountant (Electron Microscopy Sciences, Fort Washington, PA).

2.3.4 Analysis

AR-ir: Quantitative analysis was performed to analyze numbers of AR-ir neurons in standard areas of analysis in POA (1000µm x1000µm), VMH (1000µm x1000µm), BNST (500µm x 1750µm), medial amygdala (500µm x 1750µm), and ARC (1000µm x1250µm; rostral and middle levels (*20, 23*)), Counts were performed in 3-6 hemi-sections per animal per region, using Neurolucida software (Microbrightfield Inc.) attached to a motorized Leica DM5000B (Leica Microsystems). Number of AR-ir neurons were averaged per animal and compared between treatment groups, within each brain region, using one-way ANOVA and Dunn's or Holm-Sidak method post hoc analyses, with 95% confidence levels (SigmaStat version 3.0.1). In experiment 1A,

numbers of AR-ir neurons were compared between control males, control females, prenatal T females, and castrated males; in experiment 1B, AR-ir was compared between control and prenatal T females (using same tissues as in experiment 1A), prenatal DHT and E-treated females. Finally, in experiment 2, control and prenatal T-treated females were compared to F or TF females.

Colocalization AR/kiss or AR/AgRP neurons: All kisspeptin (in POA and ARC) and AgRP neurons (in ARC) were analyzed for colocalization of AR using a LeicaDM5000B at 20X magnification, in 3-4 hemisections per animal. Percentages of kisspeptin or AgRP neurons that colocalized AR were calculated for each section and averaged per animal. Comparisons between treatment groups were conducted within each brain region, using ANOVA, and Dunn's or Holm-Sidak, with 95% confidence levels.

2.3.5 Images

All images were captured using Neurolucida software (Microbrightfield Inc.) attached to a motorized Leica DM5000B (Leica Microsystems) microscope and Leica DFC420 camera. Figures were prepared using Adobe Photoshop CS2. Images were not altered in any other way, except for occasional adjustment of brightness.

2.4 Results

2.4.1 Effects of prenatal T treatment on AR immunoreactivity (ir)

In experiment 1a, a sex difference and effect of prenatal T treatment were observed in AR-ir in POA, ARC, VMH, BnST, and MeA ($F(3,19)$:28.6-63.9, $p<0.05$-<0.001) Figures 9 and 10. Control males had more AR-ir neurons in all areas compared to control females ($p<0.001$), and castration significantly reduced AR-ir in all areas ($p<0.001$) to levels comparable to control females. Prenatal T treated females had more AR-ir neurons compared to control females in the POA ($p<0.001$) and ARC ($p<0.001$), but not in the VMH, BnST, or MeA. However, prenatal T treatment did not increase AR-ir to male levels, as AR-ir was significantly lower in all areas compared to control males ($p<0.001$), although significantly higher than castrated males in the POA ($p<0.001$), ARC ($p<0.001$) and VMH ($p=0.009$).

To confirm that prenatal T organizes AR-ir in adult ewes via androgenic actions, AR-ir in POA and ARC in the same control and prenatal T females was also compared with females treated prenatally with DHT or E (experiment 1b; $F(3,16)$:21.56-37.87, $p<0.001$). Prenatal DHT treated females had similar expression of AR-ir compared to prenatal T females, and were significantly higher than control females ($p<0.001$). In contrast, prenatal E treatment did not affect AR-ir (Table 1).

Next (experiment 2), it was determined if the effects of prenatal T on AR-ir are reversed by co-treatment with flutamide, and hence are via blocking the androgenic actions of T. Prenatal T treated females had a significantly higher number of AR-ir neurons in the ARC ($F(3,18)$:10.21; $p=0.004$) and POA ($F(3,19)$:15.22; $p<0.001$) compared to control

females, confirming results from experiment 1. Furthermore, co-treatment with F attenuated the effects of prenatal T, as AR-ir in TF females were similar to control females and significantly lower compared to prenatal T females ($p<0.001$). Finally, F treatment alone had no effect on numbers of AR-ir neurons (Fig. 10).

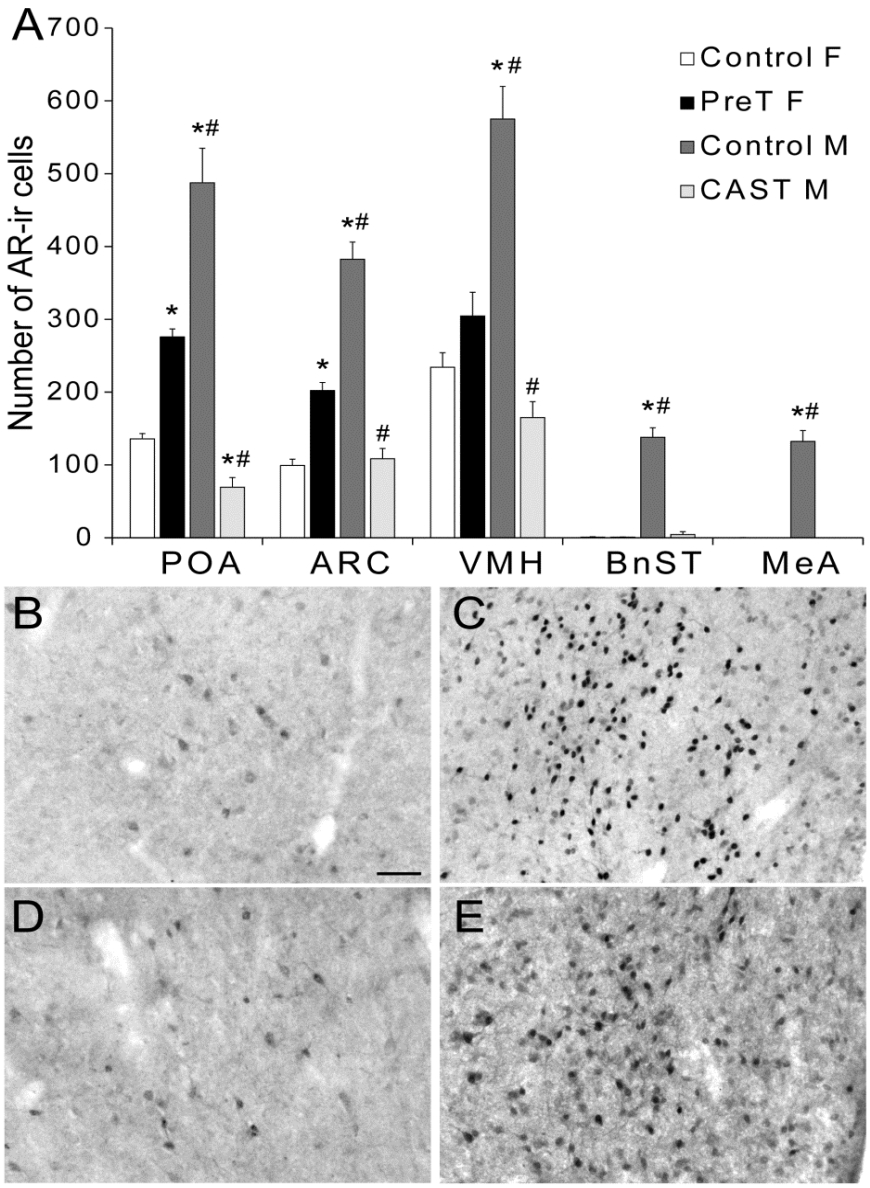

Figure 9. A) Mean ±SEM number of AR-immunoreactive (ir) neurons in the preoptic area (POA), arcuate (ARC; rostral and middle levels (*20*, *23*), ventromedial hypothalamus (VMH), bed nucleus of the stria terminalis (BnST), and medial amygdala (MeA) compared between control females, prenatal T females, control males, and castrated males. * describes significant difference from controls, # describes significant difference from prenatal T. Representative images of AR-immunoreactive neurons in the ARC of B) control female, C) control male, D) castrated male, and E) prenatal T female. Scale bar represents 50µm.

Brain region	Number of AR-ir neurons			
	Control	**Prenatal T**	**Prenatal DHT**	**Prenatal E**
POA	135.9±7.1	275.8±11.0*(p<0.001)	239.5±21.3*(p=0.004)	110.9±9.3
ARC	86.2±6.3	177.3±15.6*(p<0.001)	198.5±10.3*(p<0.001)	63.3±15.0

Table 1.Mean ±SEM number of AR-immunoreactive neurons in the preoptic area (POA) and rostral arcuate (*20, 23*) (ARC) of control, prenatal testosterone (T), prenatal dihydrotestosterone (DHT), and prenatal estradiol (E) treated females. * describes significant difference from controls.

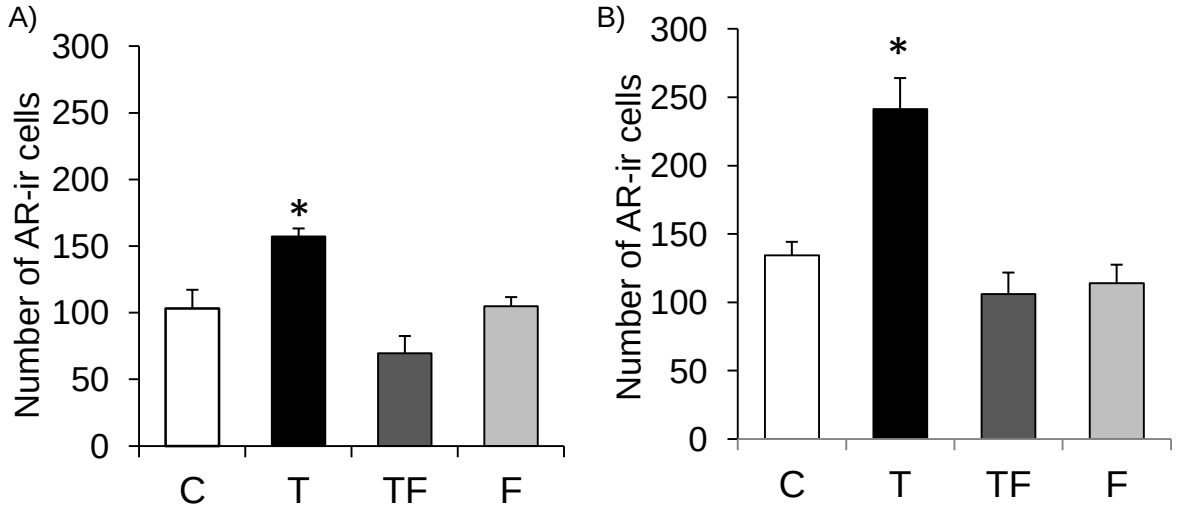

Figure 10. Mean ±SEM number of AR immunoreactive cells in the arcuate nucleus (A) and preoptic area (POA) (B) compared between control, prenatal testosterone (T), prenatal flutamide (F) and prenatal TF females.

2.4.2 Effects of prenatal T treatment on AR immunoreactivity in kisspeptin or AgRP neurons

Prenatal T treatment significantly increased AR expression in kisspeptin neurons in the ARC ($F(3,18):19.87$, $p=0.001$) and POA ($F(3,19):12.72$, $p<0.001$) as well as ARC AgRP neurons ($F(3,18):19.60$, $p<0.001$), compared to control females. Furthermore, effects of prenatal T treatment were attenuated by co-treatment with Flutamide (TF), as expression of AR-ir in POA kiss, ARC kiss, and ARC AgRP neurons in TF-treated females was significantly lower compared to prenatal T females ($p<0.001$), and comparable to control or F-treated females (Fig. 11). In addition, total numbers of AgRP and kisspeptin neurons were counted and compared between groups. Prenatal T treated females had significantly more AgRP-ir neurons compared to control females ($p=0.001$) and prenatal TF treatment was able to reverse this effect ($p=0.06$). Numbers of kiss neurons did not differ between groups. These results are consistent with our previous findings (*20, 23*).

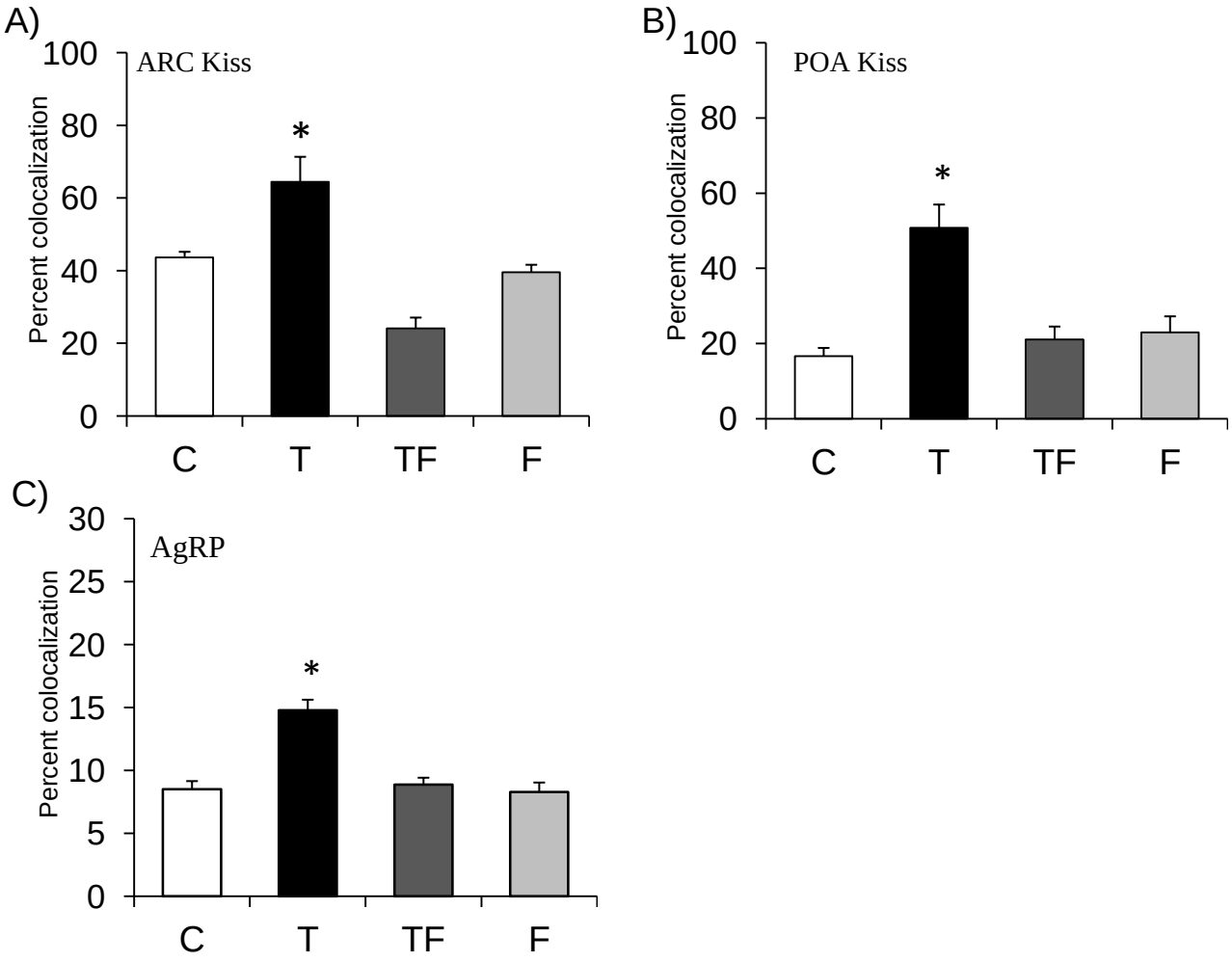

Figure 11. Mean ±SEM percent colocalization of AR with kisspeptin neurons in the arcuate nucleus (ARC) (A) and preoptic area (POA) (B) and AgRP neurons in the ARC (C) compared between control, prenatal testosterone (T), prenatal flutamide (F) and prenatal TF females.

2.5 Discussion

In this study, we examined the expression of AR-immunoreactive (ir) neurons in the hypothalamus and other limbic regions of the sheep brain and describe sex differences in AR-ir as well as alteration in AR-ir as a result of prenatal T treatment. Finally, we show an increase in AR-ir with ARC and POA kisspeptin and ARC AgRP neurons as a result of prenatal T treatment.

Specifically, we show that adult females, prenatally exposed to T show an increased number of AR-ir neurons in the POA and ARC and a trend towards more AR-ir neurons in the VMH. Although adult rams showed the highest AR-ir in all brain areas analyzed, prenatal T treated females showed more AR-ir neurons than CAST rams. Throughout the prenatal period, testosterone increases androgen receptor AR expression in males resulting in greater AR density in males compared with females (*31*). Scott *et al.* (2004) also found a sex difference in the level of AR-mRNA containing neurons, with a greater level of AR mRNA expression in the medial preoptic area and BNST of rams compared with ewes (*32*). These sex differences, along with the effects of prenatal T treatment suggest a masculinization of the female brain by T and more than likely influence reproductive and metabolic neuroendocrine function and behavior in adulthood. Indeed, prenatal T induced changes in AR-ir were mimicked by prenatal dihydrotestosterone (DHT), but not estradiol (E) treatment and reversed by prenatal co-treatment with the androgen receptor blocker, flutamide, strongly suggesting that these changes were programmed via the androgenic actions of T.

As a reliable and functional model, ewes exposed to T from prenatal days 30-60 show various reproductive and metabolic deficits manifested as early reproductive failure and oligo-ovulation (*7, 33-36*), neuroendocrine feedback defects (*6, 34, 37-40*), and decreased insulin sensitivity (*13, 22*). Moreover, these females display altered sexual behavior exhibited male-typical mating behaviors directed at estrous females and aggression towards males (*14*). In this study we show that like rams, prenatal T treated ewes have significantly more AR-ir neurons in the ARC and POA and show a strong trend for increased AR-ir neurons in the VMH compared to control females. In the sheep, these areas are critical in the control of metabolic (*41-43*) and reproductive (*18-19, 21, 44-47*) neuroendocrine functions as well as proceptive and receptive sexual behavior (*48*), respectively. As such, early life alterations in neuroendocrine functions and masculinization of sexual behavior in the sheep are most likely programmed during a critical period of prenatal development and/or maintained by high levels of circulating T during adulthood.

Although control rams showed the highest AR-ir in all brain areas analyzed, castrated rams had comparable or lower numbers of AR-ir neurons than control ewes. It is difficult to say whether the increase in AR-ir that we observe in the adult sheep brain is an indication of circulating testosterone or an organizational effect of exposure to T during a critical period. Indeed, removal of the gonads (ovariectomy or castration) eliminates a main source of testosterone in both rams and ewes. Therefore, since control ewes and castrated rams (naturally exposed to T prenatally) have comparable low AR-ir and prenatal T treated ewes have increased AR-ir, this suggests an activational effect of

testosterone during adulthood. However, we cannot dismiss the role of the adrenal glands in the production of circulating androgens (*49*), which may be sexually dimorphic. In fact, it has been shown that during fetal life, female sheep show an increased rate of androgen synthesis from the adrenals when compared to males (*50*). Furthermore, prenatal T exposure programs both ovarian (*51*) and adrenal (*52*) hyperandrogenism in the ewe. Nevertheless, adrenalectomy is not plausible in our current sheep model, leaving this question to be addressed in future studies.

Next, we focused on two neuronal populations found in the ARC and POA, the kisspeptin and AgRP neurons, and found an increase in AR colocalization with both populations. This increase was not seen in prenatal T females that were co-treated with the androgen blocker, flutamide (TF), suggesting that prenatal T acts via an androgenic mechanism to lay the framework for these changes seen during adulthood. The ARC kisspeptin subpopulation (also known as the KNDy neurons) have been shown to play an important role in the surge and pulsatile secretion of GnRH, while POA kisspeptin neurons have been shown to be critical for the GnRH ovulatory surge (*17, 19, 21, 53-54*). Prenatal T treated female sheep show increased GnRH/LH pulsatile (*55-56*) secretion and a dampened and delayed GnRH/LH ovulatory surge (*6, 56*), both parameters which can be mediated via kisspeptin neurons. Moreover, circulating testosterone levels have been shown to differentially mediate KNDy neuropeptide expression. In the male mouse, testosterone acts through both AR and ERα to inhibit *Kiss1*(encoding kisspeptin) (*57*) and *Pdyn* (encoding Dynorphin) mRNA expression (*58*) in ARC KNDy neurons. However, in

other regions that contain kisspeptin neurons such as the anteroventral periventricular nucleus (AVPV), T stimulates the expression of *Kiss1* mRNA. These findings show that kisspeptin neurons are direct targets for T, which likely plays a role in mediating both pulsatile and surge mechanisms. Furthermore, Navarro *et al.* (2011) found that AR knockout *(*KO) mice had higher levels of *Kiss1, Tac2 (encoding NKB),* and *Pdyn* mRNA in the ARC compared to wild type controls, suggesting that AR plays a role during the developmental period of the of KNDy neurons (*59*), possibly regulating peptide expressing during this sensitive period (*60*). Previous work from our lab showed that prenatal T treatment decreases the number of dynorphin and NKB, but not kisspeptin immunoreactive neurons in the ARC of the ewe (*20*), providing further support for the lasting effects of T on the KNDy neurons.

In this study, we show that prenatal T treated females had higher AR colocalization with arcuate AgRP neurons, orexigenic neurons which signal increased food intake and decreased energy expenditure (*61*). Our lab has previously shown that prenatal T treatment increases the number of AgRP neurons to more than double the amount seen in control ewes and co-treatment with flutamide (TF) significantly lowers AgRP neurons numbers, suggesting that this change is largely programmed via the androgenic actions of T (*23*). These prenatal T females also show a decrease in insulin receptor (IR) colocalization in the same neurons (*62*). Although much less is known about the role of testosterone in the regulation of AgRP expression, the increase in AR colocalization with these neurons may reflect a means by which testosterone acts to modulate these changes. In castrated male rats, T replacement increases ARC neuropeptide Y (NPY) mRNA, an

orexigenic neuropeptide which is highly colocalized with AgRP (*23, 63*), suggesting regulation of this peptide by T (*64*). One possibility is that early life exposure to prenatal T, acting via AR, alters AgRP and IR expression in this population and contributes to the insulin resistance displayed by out prenatal T sheep (*13, 22*). This is supported by the finding that prenatal TF treatment blocks the increase in AR and decreased IR colocalization with AgRP neurons, as well as the increase in AgRP neuronal number (*23*). These findings suggest an androgenic of T action on the ARC AgRP population; however the mechanisms which program these alterations merit further investigation.

In summary, there are well documented metabolic and reproductive deficits stemming from excess exposure to T during the prenatal period of development in the sheep. The present study shows that prenatal T treatment increases AR-ir in areas of the ewe brain that are critical in the control of reproductive and metabolic functions as well as sexual behavior. However, the mechanism by which prenatal T alters postnatal development of these systems is not yet known. One possibility is that prenatal T masculinizes the female brain, rendering it more sensitive to circulating androgens and thus, altering control of neuroendocrine functions in the adult female. Prenatal T exposure may also result in abnormal programming of androgen producing tissues, such as the ovaries and adrenals, resulting in increased circulating androgens (*51-52*). Therefore, the increased AR-ir seen in prenatal T females could be a programmed prenatal effect and/or a reflection of high levels of circulating androgens. Nevertheless, this study is the first to provide a measure of androgenism in the brain of the prenatal T treated sheep, therefore confirming hyperandrogenism in this model.

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Chapter 3: Prenatal Testosterone Decreases Colocalization of Insulin Receptors in KNDy and AgRP neurons in Adult Ewes: Implications for Reproductive and Metabolic Dysfunction

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3.1 Abstract

Insulin serves as a link between the metabolic and reproductive systems, communicating energy availability to the hypothalamus and enabling reproductive mechanisms. Prenatal testosterone (T)-treated Suffolk sheep display an array of reproductive and metabolic dysfunctions including insulin resistance, similar to those of women with polycystic ovarian syndrome (PCOS). Prenatal T-treated sheep show alterations in KNDy (co-expressing kisspeptin, neurokinin B/dynorphin) and agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC), two populations that play key roles in the control of reproduction and metabolism, respectively. In this study, we examined if prenatal T-treatment perturbed insulin receptor localization in preoptic area (POA) kisspeptin neurons, ARC KNDy neurons, agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), and gonadotropin-releasing hormone (GnRH) neurons. Analysis of immunohistochemical visualization of the beta subunit of insulin receptor (IRβ), revealed that KNDy, AgRP and POMC, but not GnRH or POA kisspeptin neurons, colocalize IRβ. Moreover, prenatal T treatment decreased percentages KNDy and AgRP neurons that colocalized IRβ, consistent with reduced insulin sensitivity. Co-administration of the anti-androgen flutamide during prenatal T treatment prevented the reduction in IRβ colocalization in AgRP, but not in KNDy neurons, indicating that the effects of prenatal T on these two populations are programmed differentially by androgenic and estrogenic actions of T, respectively. These findings indicate that defects in insulin signaling at the level of the brain likely contribute to the insulin resistance of this PCOS model.

3.2 Introduction

Fertility is dependent on sufficient energy supply. Insulin is a key communicator of energy availability to the hypothalamus and specifically gonadotropin-releasing hormone (GnRH) neurons, enabling reproductive function in times of sufficient energy supply (1). In women with polycystic ovary syndrome (PCOS), infertility and insulin resistance are major concerns and are exacerbated by excess weight (2-5). The sheep model of PCOS, in which ewes are exposed to excess T during fetal life from day 30–90 of gestation, replicates the reproductive and metabolic deficits seen in women with PCOS (6-8). The reproductive disruptions include intermittent to absent ovulation (9-12), early reproductive failure (9-13), neuroendocrine feedback defects (14-19), and functional hyperandrogenism (20). In addition, prenatal T-treated sheep are metabolically compromised and manifest reduced insulin sensitivity and compensatory hyperinsulinemia (21-22). Although the reproductive and metabolic outcomes of prenatal T exposure are well described in the ewe, the mechanisms responsible for these detrimental effects, and the neural contribution to these deficits, are not well understood.

In sheep and other mammals, the arcuate nucleus (ARC) of the hypothalamus is a major center of convergence for reproductive and metabolic signals. This region contains two key populations of appetite regulating neurons, the orexigenic agouti-related peptide (AgRP) and the anorexigenic proopiomelanocortin (POMC) neurons. Previous work from our lab has shown that both AgRP and POMC neurons express insulin receptors (82% and 92%, respectively, Lehman M.N. preliminary data) and prenatal T-treated females

show an increase in both AgRP-immunoreactive neurons and fiber density in the ARC (23). In addition to the metabolic control neurons, the ARC also contains a key neuronal group involved in the control of GnRH secretion, the KNDy (co-expressing the neuropeptides kisspeptin, neurokinin B (NKB), dynorphin) neuronal population. Kisspeptin, NKB and dynorphin have each been proposed to serve distinct roles in modulating GnRH release depending on the steroid hormone milieu, mediating pulsatile and/or surge secretion of GnRH (24-29). Previous work has found that prenatal T-treated females show a reduction in dynorphin and neurokinin B immunoreactivity in KNDy neurons (30), but no change in kisspeptin (30). This peptide imbalance has been postulated to contribute to the steroid feedback dysregulation and reproductive deficits seen in prenatal T-treated females. In addition to these findings, it has been shown that insulin sensitizer treatment improves reproductive function in prenatal T-treated females (31) as in women with PCOS (32-33), suggesting that insulin signaling is an important component of the PCOS phenotype, and may have impact on both metabolic and reproductive functions. There is evidence from rodent studies that insulin can have significant effects on reproduction, acting at a neural level. For example, diabetic female rats show decreased luteinizing hormone (LH) surges and fewer ovulatory cycles (4). Moreover, deletion of neuronal insulin receptors in mice results not only in increased food intake, body weight, diet-sensitive obesity, and insulin resistance, but also infertility (*1, 34*). Restoration of insulin receptor expression in the brain is required to maintain fertility in these females (35). These actions may be mediated by direct insulin action on

GnRH neurons or by input from afferent insulin sensitive neurons, as GnRH neurons have not yet been shown to express insulin receptor *in vivo*.

In this study, we examined whether insulin has the potential to act directly on KNDy and GnRH neurons in the adult sheep brain by determining if immunoreactivity for insulin receptors is present in these neuronal populations. In addition, we hypothesized that percentage of insulin receptor colocalization in these neuronal populations, and/or in AgRP and POMC neurons, would be altered by exposure to excess prenatal T. Because insulin appears to play an important role in relaying energy availability to the reproductive axis, we predicted that prenatal T-treated ewes would show a decreased expression of insulin receptors in metabolic and reproductive neurons of the ewe, rendering these neurons less sensitive to insulin and thereby affecting processes that require specific information about energy availability.

3.3 Materials and Methods

3.3.1 Animals and experimental groups

Suffolk ewes were cared for at the University of Michigan Sheep Research Facility (Ann Arbor, MI). Details of housing, nutrition, breeding, lambing and prenatal treatment have previously been described (*12, 36-37*). Prenatal treatment involved administration of hormones to the pregnant mother between days 30 and 90 of gestation of a 147 day gestation period. The three experimental groups used in this study consist of adult female offspring treated: prenatally with T (T group; n=5), with T and flutamide (TF; n=5), or flutamide alone (F; n=5). Control females (n=5) received an equal volume of vehicle (2 ml cottonseed oil). If twin births occurred, only one offspring from the pair was included. All procedures conducted were approved by the Institutional Animal Care and Use Committee of the University of Michigan and were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

T (T-propionate; Sigma-Aldrich, St. Louis, MO) was injected i.m. twice weekly (100 mg/2ml dissolved in cottonseed oil). Previous analysis of maternal and fetal blood circulation has demonstrated that this dosage of T produces levels similar to adult males and fetal males, respectively (38). Females in the F group received daily injections of flutamide (15mg/kg, Sigma-Alderich) dissolved in dimethylsulfoxide (400mg/ml, Sigma-Aldrich) administered subcutaneously. Females in the TF group received T-propionate as described above in addition to daily injections of flutamide.

3.3.2 Tissue Collection

Brains were collected during the breeding season when females were 2 years of age. Three-four weeks prior to tissue collection, animals were ovariectomized and were treated sequentially for 11–12 d with two controlled internal drug release P_4 implants (CIDR) (InterAG, Hamilton, Waikato, New Zealand) and then for 1day with four 3-cm-long E_2 implants to simulate ovarian steroid levels during the late follicular phase of the cycle. Eighteen hours after the E2 implants, animals were sacrificed. At time of tissue collection, all ewes received two intravenous injections (at 10 min intervals) of 25,000 U heparin (catalog # 402588B; Abraxiz Pharmaceuticals, Schumberry, IL). Ewes were anaesthetized intravenously with sodium pentobarbital (390 mg/ml/kg; Fatal Plus, Vortech, Dearborn, MI) and rapidly decapitated. The heads were immediately perfused through the internal carotid artery with 6L of 4% paraformaldehyde (Sigma-Aldrich) dissolved in 0.1M phosphate buffer (PB; Sigma- Aldrich) containing 0.1% sodium nitrate (Sigma- Aldrich), and 10 U/ml heparin (Abraxiz Pharmaceuticals). Brains were submerged in the same fixative at 4°C for 18hr. Blocks of tissue containing hypothalami were transferred in 30% sucrose (Caledon, Georgetown, Ont., Canada) at 4°C and sectioned coronally (45 µm) using a freezing microtome (Microm, Walldorf, Germany) into 12 parallel series for each animal. Sectioned tissues were stored in cryoprotectant (30% ethylene glycol, 0.1% sodium azide, 30% sucrose in PB) at -20°C until further processing.

3.3.3 Immunohistochemistry: general

One series of every sixth section containing ARC, POA and MBH from each animal were processed for immunohistochemistry. All incubations occurred at room temperature with gentle agitation. Free-floating sections were rinsed thoroughly with 0.1 M phosphate-buffered saline (PBS) between incubations. Antibodies were dissolved in incubation solution consisting of 0.1M PBS, 0.4% Triton X-100 (catalog item BP151- 500; Sigma-Aldrich) containing 4% normal goat serum (NGS) (catalog item 005-000-121; Jackson Immuno Research Laboratories, West Grove, PA). Unless otherwise specified, tissue sections were washed extensively with 0.1 M PBS (pH 7.35) between steps. Prior to incubation with first primary antibody sections were incubated with 1% hydrogen peroxide (10 min, H_2O_2; catalog item H325; Fisher Scientific, Pittsburgh, PA) and incubation solution (1hr) to prevent non-specific background labeling. See Table 1 (Supplemental Material) for detailed information on all antibodies used.

3.3.4 Immunohistochemistry: triple-label immunofluorescence for AgRP/IRβ/POMC

Sections were incubated overnight (17 hours) with antibody specifically recognizing AgRP (raised in Guinea Pig, 1:800 dilution in incubation solution with 4% NGS; Antibodies Australia, catalogue # GPAAGRP.1, Lot # AS506), and with goat anti-guinea pig Alexa 488 (1:100 dilution, 30 minutes, Molecular Probes, Inc., Carlsbad, CA). Next, sections were incubated overnight with antibody recognizing the beta sub-unit of the insulin receptor (IRβ, raised in Rabbit, 1:300 dilution, Santa Cruz, C-19, SC-711), with biotinylated goat anti-rabbit (1:500 dilution, 1 hour, Vector Laboratories, Burlingame,

CA, USA), ABC-elite (1:500 dilution in PBS, 1 hour, Vector Laboratories), biotinylated tyramide (BT; 1:250 dilution in PBS with 1 µl of 3% H_2O_2/ml, 10 minutes, Perkin Elmer Life Sciences, Woodbridge, Ont., Canada), and Alexa 555 conjugated streptavidin (1:100 dilution in PBS, 30 mins., Molecular Probes). Finally, sections were incubated with antibody specifically recognizing POMC (raised in rabbit, 1:4,000 in incubation solution with 4% normal donkey serum, Phoenix Pharmaceuticals, Burlingame, CA, USA, catalogue # H-029-30) and donkey anti-rabbit Cy5 (1:100 dilution in PBS, 30 minutes, Molecular Probes). Sections were mounted on plus charged slides and cover slipped with gelvatol (39).

Specificity of primary antibodies for POMC and AGRP has previously been demonstrated in sheep hypothalamic sections (39). Pre-absorption of IRβ antibody with its corresponding peptide antigen (Santa Cruz) at concentration of 10 µg/ml for 24 hours at 4°C eliminated all immunoreactive staining (Supplemental Fig. 1). Finally, elimination of any single primary antibody while performing all other steps of the protocol eliminated all staining for the corresponding antigen and no others, demonstrating lack of cross-reactivity.

3.3.5 Immunohistochemistry: dual-label immunofluorescence for kisspeptin/GnRH with IRβ

Sections were incubated overnight with antibody specifically recognizing IRβ (raised in rabbit, 1:300 dilution, Santa Cruz, C-19, SC-711). Next sections were incubated with biotinylated goat anti-rabbit (1:500 dilution, 1 hour, Vector Laboratories, Burlingame,

CA, USA), ABC-elite (1:500 dilution in PBS, 1 hour, Vector Laboratories), biotinylated tyramine (BT; 1:250 dilution in PBS with 1 µl of 3% H_2O_2/mL, 10 minutes, Perkin Elmer Life Sciences, Woodbridge, Ont., Canada), and Alexa 555 conjugated streptavidin (1:100 dilution in PBS, 30 minutes, Molecular Probes). Finally, sections were incubated overnight with antibody specifically recognizing kisspeptin (raised in rabbit, 1:200,000 dilution, 17 h; kp10, lot 564; gift from Dr. Alain Caraty, Nouzilly, France) or antibody specifically recognizing GnRH (raised in Mouse, 1:400 dilution, 17 h, Sternberger Monoclonals, Inc., catalogue #SMI-41R, Lot #3). The next day, sections were washed and incubated with either goat anti-rabbit or goat anti-mouse Alexa 488 (1:100 dilution, 30 mins., Molecular Probes). Sections were mounted on plus charged slides and cover slipped with gelvatol (39).

3.3.6 Analysis

Colocalization of IRβ-immunoreactivity (ir) within AGRP, POMC, kisspeptin, and GnRH neurons was examined by confocal microscopy (as previously described in (40)). Using a Zeiss LSM-510 laser-scanning confocal microscope system (Zeiss, Heidelberg, Germany), images of the arcuate nucleus of the hypothalamus (ARC), preoptic area (POA) and medial basal hypothalamus (MBH) were captured in Z-stacks comprised of 1 µm optical sections. Alexa 555 fluorescence (IRβ) was imaged with a HeNe1 laser and a 543 nm emission filter. Alexa 488 fluorescence (kisspeptin, GnRH or AgRP) was imaged with an Argon laser and a 488 nm emission filter. Cy5 fluorescence (POMC) was imaged with a HeNe2 laser and a 633 nm emission filter. Cell counts were performed in sections through the POA at the level of the organum vasculosum of the lamina terminalis (3

sections/animal), the middle ARC at the level of the tuberoinfundibular sulcus (5 sections/animal), and the MBH at levels of the rostral to middle arcuate nucleus (3 sections/animal). In the ARC, between 30-60 neurons were examined for co-localization of IRβ for each phenotype (kisspeptin, AgRP, POMC) per animal (see Table 2, Supplemental Material). For POA and MBH sections, every single GnRH (POA and MBH) and kisspeptin (POA) neuron was analyzed for co-localization. Percentages of the kisspeptin, GnRH, AgRP or POMC-ir neurons that colocalize IRβ-ir were calculated per animal. Differences between groups were determined using Two Way Analysis of Variance. For pairwise comparisons between treatment groups, Dunn's Method or Fisher LSD Method post hoc tests were performed. 95% confidence levels were applied to all tests.

3.4 Results

3.4.1 Colocalization of AgRP and POMC with IR: effects of prenatal T

In the ARC, 85% ± 1.46 of AgRP and 92% ± 2.01 of POMC neurons colocalized IRβ (Figs.12 and 13). Prenatal T treatment significantly decreased IRβ colocalization within AgRP ($F(3,22):4.35$; C versus T: $p=0.003$), but not POMC neurons (Figure 13). Co-treatment with androgen antagonist blocked the effects of prenatal T, with prenatal TF treated females showing a similar percentage of IRβ colocalization with AgRP neurons as controls and a significantly greater percentage of colocalization than prenatal T females ($p=0.015$). Androgen antagonist treatment alone had no effect on the percentage of IRβ colocalization in either AgRP or POMC neurons, relative to controls (Figure 13).

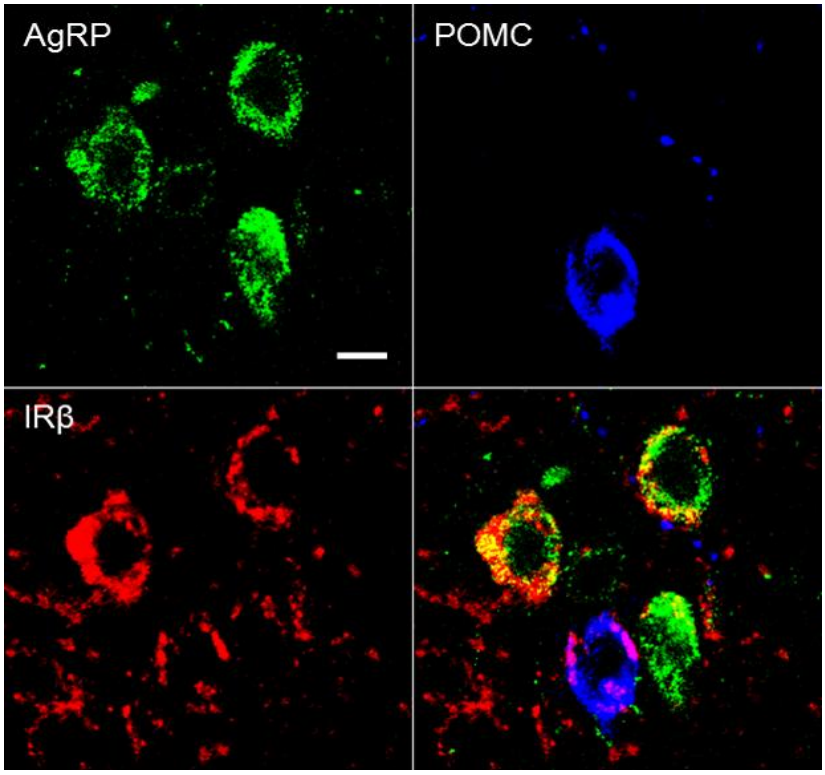

Figure 12. Triple-label fluorescence immunolabeling of AgRP- (green), POMC- (blue), and IRβ-immunoreactive neurons (red) in the arcuate nucleus of a control ewe. Overlay image (bottom right) shows colocalization of IRβ with AgRP and POMC neurons. Scale bar = 10μm.

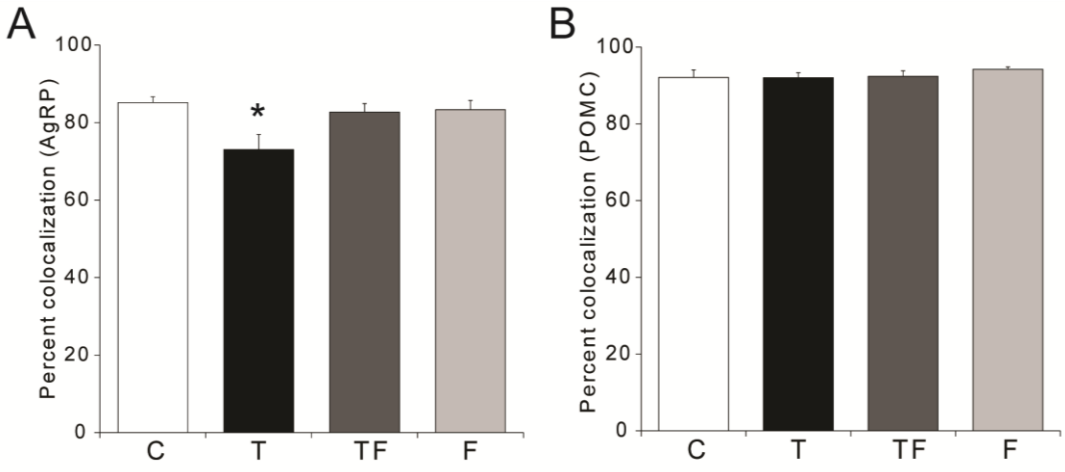

Figure 13. Mean ± SEM percentage co-localization of AgRP (A) and POMC (B) with IRβ in the arcuate nucleus of control (n=5), prenatal T (n=5), prenatal TF (n=5), and prenatal F (n=5) ewes. * indicates significant difference ($p<.05$) from controls.

3.4.2 Colocalization of kisspeptin and GnRH with IR: effects of prenatal T

In the ARC, 94% ±1.3 of kisspeptin neurons colocalized IRβ (Fig. 14, bottom panel, and 16). In contrast, POA kisspeptin neurons (104 analyzed) in control animals (n=5) do not colocalize IRβ (Fig. 14, top panel). Prenatal T treatment significantly decreased the percentage of IRβ colocalization in ARC kisspeptin neurons ($F(3,19):9.07$; C versus T $p=0.001$; Figure 16). Co-treatment with androgen antagonist did not reverse the effects of T on IRβ colocalization within ARC kisspeptin neurons, while females treated with androgen antagonist alone showed a similar percentage of colocalization as control females (Fig. 16). Finally, while immunoreactive IRβ was expressed in areas of the POA and MBH where GnRH neurons were located, out of over 300 GnRH neurons examined in 5 control animals, none colocalized IRβ (Fig. 15).

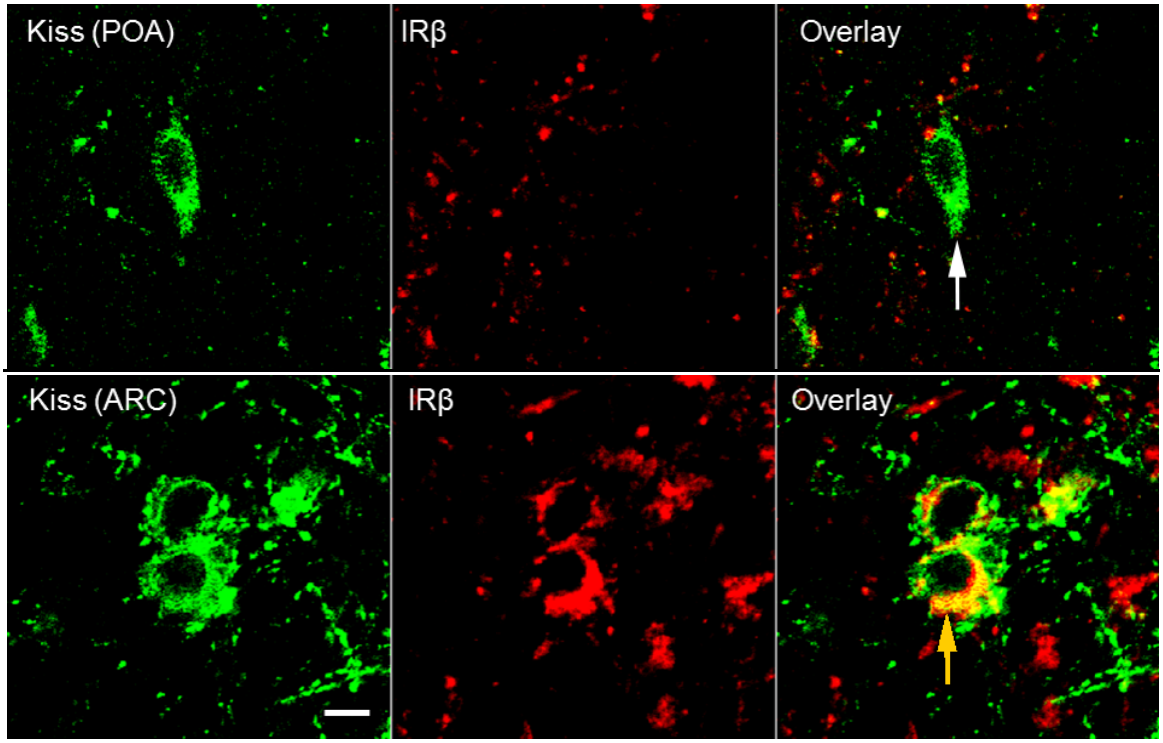

Figure 14. Dual-label immunofluorescent detection of kisspeptin- (green) and IRβ-immunoreactive (red) neurons and fibers in the arcuate nucleus (ARC) and preoptic area (POA) of the ewe. Dual-labeled kisspeptin/IRβ neuron in the ARC (yellow arrow) and a single-labeled kisspeptin (white arrow) neuron in the POA are indicated in the overlay images. Scale bar = 10µm.

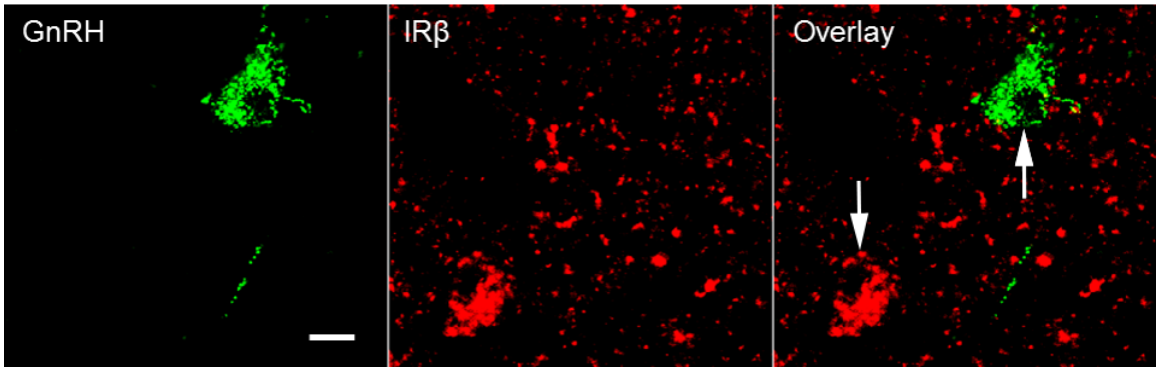

Figure 15. Dual-label immunofluorescence detection of GnRH- (green) and IRβ- (red) positive neurons in the medial basal hypothalamus of the ewe. Single labelled neurons are indicated by white arrows. Scale bar = 10µm.

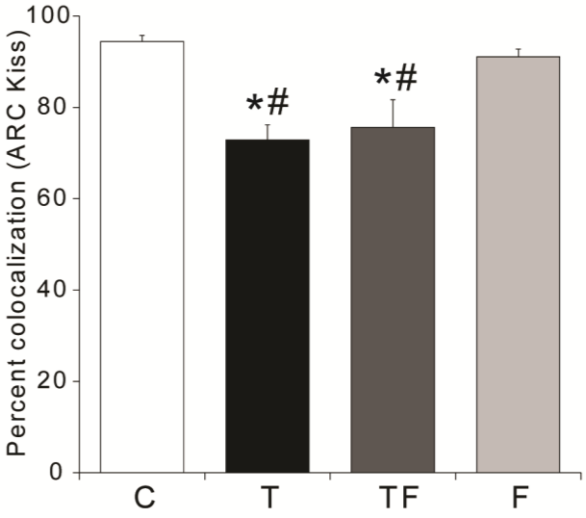

Figure 16. Mean ± SEM percentage co-localization of kisspeptin with IRβ in the arcuate nucleus of control (n=5), prenatal T (n=5), prenatal TF (n=5), and prenatal F (n=5) ewes. # indicates significant difference ($p<0.05$) from control, * indicates significant difference ($p<0.05$) from prenatal F group.

3.5 Discussion

Findings from this study document that IRβ is colocalized within ARC kisspeptin (KNDy), AgRP and POMC neurons, but not within GnRH and POA kisspeptin neurons and prenatal T treatment decreased colocalization of IRβ in AgRP and KNDy, but not POMC, neurons. Importantly, co-treatment with androgen antagonist blocked the effects of prenatal T on IRβ colocalization with AgRP, but not KNDy neurons, indicating that these opposing effects may be organized by different actions of T, androgenic and estrogenic, respectively.

3.5.1 Effects of prenatal T treatment on IR colocalization in AgRP and POMC neurons

The results of this study provide evidence of long-term changes in the colocalization of insulin receptors within AgRP neurons as a consequence of prenatal exposure to excess T. Prenatal T treatment significantly decreased the percentage of AgRP, but not POMC, neurons co-localizing IRβ in the ARC compared with controls (Fig. 13). The AgRP-specific decrease in IRβ is consistent with our previous findings showing increased numbers of AgRP-ir neurons, but not POMC-ir neurons in prenatal T-treated females (23). Since insulin decreases AgRP mRNA levels (41) and peptide release (42) in normal females, the decreased IRβ colocalization within AgRP neurons may be partly responsible for the increased AgRP peptide we see in prenatal T animals (23). In turn, both reduced IR colocalization in AgRP neurons, and increased AgRP peptide expression, may be contributing factors to the metabolic and reproductive deficits seen in

these animals. Previous studies have found that prenatal T-treated females are insulin resistant and develop compensatory hyperinsulinemia as adults (21-22). Moreover, AgRP neurons may also play an important role relaying the influence of metabolic cues to the reproductive neuroendocrine system. AgRP neurons project directly to GnRH neurons in the rat (43) and inhibit reproduction by suppressing pulsatile LH release in the rhesus monkey (44). Similarly, neuropeptide Y (NPY), an orexigenic neuropeptide, which is largely colocalized with AgRP (*23, 45*), inhibits reproduction by hindering GnRH neuronal firing in mice (46-47) and tonic secretion of LH in the sheep (48-49). We do not know whether NPY peptide, like AgRP, is increased in prenatal T sheep, but it is conceivable that increased AgRP and/or NPY peptide and its release may be a means by which this population could potentially modulate GnRH release and reproductive function.

Prenatal TF treatment was able to rescue the effects of T on IR colocalization with AgRP neurons, suggesting that the decrease in IRβ in this subpopulation is organized by the androgenic actions of T. These effects of T parallel organizational effects of prenatal T on AgRP expression, albeit more completely. We previously found that co-treatment of T with androgen antagonist (TF) was able to largely, but not completely, rescue the effects of prenatal T on AgRP neuronal numbers (23). Moreover, the effects of prenatal T on insulin resistance (22) and AgRP neuronal numbers (23) are mimicked by prenatal dihydrotestosterone (DHT, a non-aromatizable androgen) treatment, further implicating an androgenic programming role of T in this population.

3.5.2 Effects of prenatal T treatment of IR colocalization in KNDy neurons

We show that approximately 94% of KNDy neurons colocalize IRβ and that prenatal T treatment results in a 21% decrease in IR colocalization in these neurons. Reproductive deficits in prenatal T-treated females include LH excess and a delayed and dampened LH surge culminating in ablation of ovulatory cycles (14-18). As noted earlier, postnatal insulin sensitizer treatment improves reproductive function in prenatal T-treated females (31). Insulin has been shown to stimulate GnRH / LH secretion *in vivo* (50) and *in vitro* (51), an effect which is mediated at the level of the hypothalamus and not the pituitary. Because GnRH neurons do not express IRβ, insulin signaling to the reproductive axis must be relayed to GnRH neurons via afferent neurons expressing this receptor. Although the specific site of insulin action is not yet known, the ARC KNDy neurons have some of the attributes for fulfilling these criteria. KNDy neurons in the sheep have been implicated in the control of both pulsatile (52-53) and surge (54) secretion of GnRH. KNDy neurons have been shown to make direct contacts with AgRP and POMC neurons in the sheep ARC (55). Furthermore, ovine KNDy neurons are activated by leptin treatment (55) albeit indirectly (56), and colocalize IRβ (this study) rendering this subpopulation responsive to metabolic signals (57). Finally, there is an array of substantial evidence in rodents that KNDy neurons play an important role in mediating metabolic control of reproduction (58-62). However, recent findings that mice with kisspeptin specific deletion of PI3K catalytic subunits show normal weight gain and normal time of puberty(63), suggests that insulin signaling via kisspeptin neurons (including KNDy neurons) is not critical for normal pubertal development or body weight

control. Although the effects of insulin on kisspeptin mRNA or neuronal activity have not been reported, insulin-like growth factor-1 (IGF-1) has been shown to increase *Kiss1* expression in the anteroventral periventricular nucleus (AVPV) of prepubertal female rats, in the presence of estradiol (64). These findings are consistent with the positive feedback role of AVPV kisspeptin neurons in the generation of the GnRH/LH surge in rodents (28), and a role of insulin signaling in the facilitation of the GnRH/LH surge has been widely described (*1, 4, 31*). Recent studies of prenatal T-treated sheep found that postnatal insulin sensitizer treatment increased the magnitude of the LH surge (65). Nonetheless, it is worthwhile to note that insulin can act at the hypothalamic, pituitary, or ovarian level to regulate follicular development (66-67). Thus, alterations in IRβ expression in KNDy neurons and/or at other levels, may each contribute to defects in reproductive dysfunctions seen in this model.

In contrast to IR co-localization in AgRP neurons, co-treatment with androgen antagonist did not rescue the effects of prenatal T on IR colocalization within KNDy neurons, suggesting that the decrease in this subpopulation is organized by the estrogenic actions of T. An alternate possibility is that the changes in IRβ are programmed by androgenic effects of T, via other intermediaries, for example those leading to an increase in gestational insulin levels (65). However, recent findings that prenatal co-treatment with insulin sensitizer fails to rescue the effects of prenatal T treatment on GnRH/LH surge (65) argue against this possibility. Nonetheless, increased insulin levels during the period

of fetal exposure to excess T, when insulin levels in the mother are also increased (65), might intensify the degree of disturbance in this model.

3.5.3 Absence of IRβ localization in GnRH neurons and POA kisspeptin neurons

In contrast to AgRP, POMC and KNDy neurons, we found no evidence of colocalization of IRβ in either GnRH neurons or POA kisspeptin neurons. The absence of IRβ colocalization with GnRH neurons suggests that metabolic signals controlling reproductive status are relayed via an upstream population of neurons. As noted above, KNDy neurons as well as AgRP neurons represent likely candidates, since both populations of neurons colocalize IRβ, have direct projections to GnRH neurons (*28, 43, 68*), and have been shown to regulate GnRH activity (*24, 28, 44, 46-47*). While we found no colocalization of IR in POA kisspeptin neurons, we cannot exclude the possibility that this subset of kisspeptin neurons may participate in these actions via inputs from other insulin-sensitive neurons.

3.5.4 Conclusion

A summary of our current knowledge of changes in insulin receptors and metabolic peptides as a consequence of exposure to prenatal T in the female sheep, and their relationship to the reproductive neuroendocrine system, is shown in Figure 17. KNDy neurons have been posited as a major intermediary conveying metabolic signals to the GnRH neuron (58-62), although direct projections of AgRP and POMC neurons to GnRH neurons are present in rodents (*43, 68-70*). As shown by the enclosed dotted circle,

KNDy neurons form a reciprocally interconnected neuronal population whose activity is hypothesized to play a key role in regulating GnRH pulsatile secretion, as well as participate in the control of the GnRH surge in sheep and primates (28). Prenatal T leads to decreased IR in KNDy neurons, and also decreased expression of two of the three KNDy peptides, neurokinin B and dynorphin (not shown). Prenatal T also results in decreased in IR in AgRP but not POMC neurons, and there is a corresponding increase in AgRP but not POMC peptide in these populations. The precise functional impact that each of these changes has on the control of GnRH secretion remains to be determined, but a first step will be to determine whether pre- or postnatal treatments with insulin sensitizers, such as Rosiglitazone, can reverse any of these receptor/peptide changes. Ultimately, the challenge will be to understand the early developmental cascade of events initiated by exposure to excess T that result in adult reproductive and metabolic dysfunction, and to determine which of the long-term hypothalamic alterations seen in the adult offspring may contribute to these functional deficits.

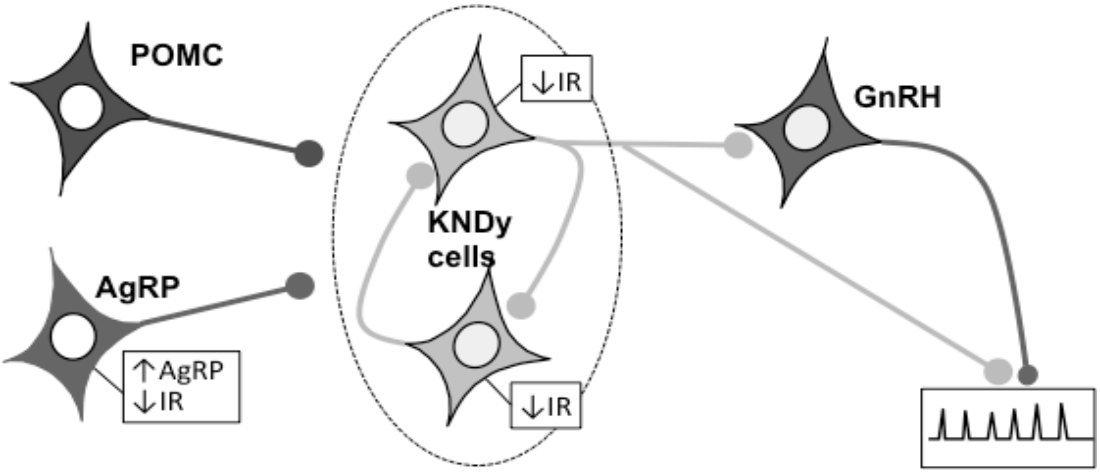

Figure 17. Schematic diagram summarizing the effects of prenatal T treatment on insulin receptor (IR) colocalization within AgRP, POMC and KNDy neurons in the adult sheep hypothalamus. Decreased IR in AgRP neurons may, in part, underlie the increased AgRP peptide in prenatal T ewes previously described (23). In turn, changes in AgRP and KNDy peptides may contribute to alterations in the steroid feedback control of GnRH secretion by way of inputs at the level of GnRH neuronal bodies or their terminals. Direct projections from AgRP neurons onto GnRH neuronal bodies are not shown.

Appendices:

Appendix 1. *IRβ Antibody Controls*

The specificity of the antibody used for insulin receptor beta (IRβ) was verified by preabsorption of the diluted primary antibody (1:300) with immunizing peptide (Santa Cruz) at a concentration 10g/ml (Fig 18), which effectively eliminated immunostaining.

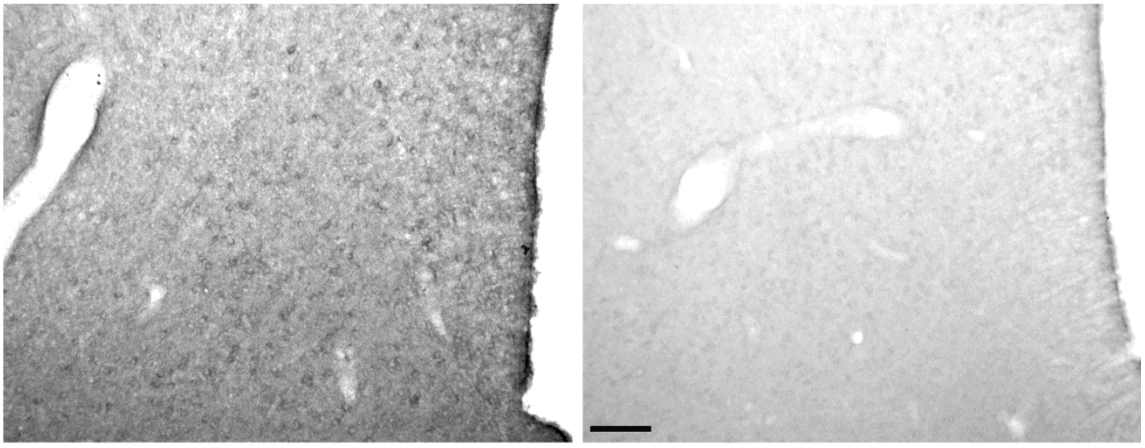

Figure 18. Insulin receptor immunoreactivity in the ovine ARC of a control ewe (A) and following preabsorption of the diluted antibody with the peptide antigen at a concentration of 10µg/ml (B). Scale bar = 100µm.

Chapter 3: References

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Chapter 4: Prenatal and postnatal contribution of insulin in the programming of androgen and insulin receptor colocalization with KNDy and AgRP cells in the hypothalamus of prenatal testosterone-treated sheep

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4.1 Abstract

Similar to women with polycystic ovarian syndrome (PCOS), prenatal testosterone (T) treated ewes develop metabolic and reproductive defects such as insulin resistance, decreased hypothalamic sensitivity to gonadal steroid feedback, and deterioration of ovulatory cycles. These hallmark features are often accompanied by hyperinsulinemia and hyperandrogenemia, which are believed to act synergistically during the prenatal and/or postnatal periods to program and maintain the metabolic and reproductive dysfunctions mentioned above. Prenatal T treated adult ewes show an increase in androgen receptor (AR) and a decrease in insulin receptor (IR) colocalization within agouti-related peptide (AgRP) and KNDy (kisspeptin, neurokinin B and dynorphin) neurons in the hypothalamic arcuate nucleus. AgRP and KNDy neurons play a key role in the control of metabolic homeostasis and reproductive function, respectively. The current study examines whether treatment with the insulin sensitizer, Rosiglitazone (R) during the prenatal or postnatal period can reverse the increased AR and decreased IR colocalization seen within AgRP and KNDy neurons in prenatal T-treated ewes. Pregnant Suffolk ewes were administered twice weekly injections of T propionate (100 mg i.m.) from days 30 to 90 of gestation. In addition, separate groups of pregnant ewes received prenatal T combined with either R (8 mg/ewe/day, oral) or F (15 mg/kg/day, s.c.). An additional group of prenatal T-treated offspring received postnatal R (0.11 mg/kg/day, oral), beginning at 8 wk of age. At 21 months of age, adult female offspring were ovariectomized and received hormonal implants to produce an artificial follicular phase prior to sacrifice, and brains were processed for immunocytochemistry. We show that

prenatal (TR), but not postnatal treatment with insulin sensitizer (T+R) treatment blocks the prenatal T induced effect on IR, but not AR colocalization, within AgRP and KNDy neurons. These findings suggest that IR expression in AgRP and KNDy neurons is prenatally programmed by a mechanism involving insulin signalling. Given the role of AgRP and KNDy neurons in the regulation of energy homeostasis and reproductive function, prenatal intervention with insulin sensitizer may alleviate some dysfunctions associated with PCOS.

4.2 Introduction

Polycystic ovarian syndrome is one of the most common endocrine disorders, affecting more than 10% of reproductively aged women (*1*). Dysfunctions associated with PCOS include hyperandrogenism, polycystic ovaries, insulin resistance, compensatory hyperinsulinemia, irregular ovulation, and infertility (*2-7*). PCOS is a complex disorder stemming from genetic as well as environmental disturbances (*8*). Nonetheless, recent experiments using animal models (*6, 9-11*), along with supporting clinical evidence (*1, 12*), have shown that exposure to excess androgens during a critical period of development can permanently disrupt metabolic and reproductive function during adulthood (*1, 8, 13-15*), resulting in a PCOS-like phenotype. Our animal model, the prenatal testosterone (T) treated ewe, allows us to study the mechanisms which are responsible for the prenatal organization of PCOS (*9*). Similar to women with PCOS, prenatal T treated sheep develop metabolic and reproductive deficits. These include insulin resistance and compensatory hyperinsulinemia (14, 15) as well as ovulatory dysfunctions (*16-17*) marked by a delayed a dampened preovulatory surge (*16*) and increased luteinizing hormone (LH) pulsatile secretion (*18*). Various studies have shown that treatment with insulin sensitizers (ie. Rosiglitazone) (*19-22*) or anti-androgens (ie. Flutamide) (*23-24*) can alleviate certain reproductive and metabolic symptoms associated with PCOS. In prenatal T treated sheep, postnatal treatment with the insulin sensitizer Rosiglitazone (R) has been shown to improve reproductive function (*25*), reducing the deterioration in number of cycles from 80% to 20% in the second breeding season.

Another study found that postnatal treatment with Flutamide (F) and R were able to reverse the effects of prenatal T on the amplitude, but not the timing, of the LH surge (*26*). Specifically, prenatal T-treated females that were treated with the anti-androgen F or insulin sensitizer (R) postnatally (T+F or T+R) exhibited an LH surge of normal amplitude, a characteristic of the preovulatory surge which is typically dampened by prenatal T treatment; despite this improvement, however, the timing of the surge was still delayed.

Prenatal interventions have also proven effective in reversing alterations seen at the level of the brain in prenatal T treated sheep. The focus of these studies has been on the arcuate nucleus (ARC) of the hypothalamus, a major center of convergence for reproductive and metabolic signals. This region contains two key populations of neurons involved in the regulation of metabolic and reproductive function, orexigenic agouti-related peptide (AgRP) neurons and KNDy (co-expressing the neuropeptides kisspeptin, neurokinin B (NKB), dynorphin) neurons. KNDy neurons are important regulators of GnRH secretion, with each of the KNDy peptides (kisspeptin, NKB and dynorphin) serving distinct roles in modulating GnRH release depending on the steroid hormone milieu, and mediating pulsatile and/or surge secretion of GnRH (*27-32*). Our lab has previously found that prenatal T treated sheep show an increase in the number of AgRP neurons (*33*) and a reduction in dynorphin and NKB, but not kisspeptin, within KNDy neurons (*34*). We also found that both AgRP and KNDy neurons of prenatal T treated sheep show an increased percentage of colocalization with androgen receptors (AR) (*35*)and a decreased

colocalization with insulin receptors (IR) (*36*). Flutamide co-administered with prenatal testosterone (TF) reverses the prenatal T induced increase in AgRP neuron number (*33*) as well as the decrease in insulin receptor (IR) colocalization in this population (*36*). However, TF treatment does not reverse the decrease in IR colocalization within KNDy neurons (*36*). Although prenatal interventions may be an effective method of reversing some effects of excess prenatal T exposure, postnatal interventions may provide another opportunity and time point where we can intervene and reverse some detrimental effects of prenatal T. In addition, recent observations of high circulating insulin levels induced by T treatment in the pregnant sheep (Abi Salloum, B., and V. Padmanabhan, unpublished data), suggest that insulin may also play a role in the programming of reproductive and metabolic functions during adulthood. Based on these observations, in this study we examined the effects of prenatal and postnatal treatments with the anti-androgen Flutamide (F) and insulin sensitizer, Rosiglitazone (R) on prenatal T induced changes in AgRP and KNDy neuronal populations and specifically, on the colocalization of AR and IR within these neurons.

4.3 Materials and Methods

All study procedures were approved by the Institutional Animal Care and Use Committee of the University of Michigan and are consistent with National Research Council's Guide for the Care and Use of Laboratory Animals. Suffolk ewes were cared for at the University of Michigan Sheep Research Facility (Ann Arbor, MI). Details of housing, nutrition, breeding, lambing and prenatal treatment have previously been described (*37-39*). Parturition of all lambs took place in early 2010 from March through April. Lactating ewes were fed 1 kg of shelled corn and 2–2.5 kg of alfalfa hay/ewe per day. Lambs were provided ad libitum with commercial feed pellets (Shur-Gain, Elma, NY) consisting of 18% of crude protein. Breeder ewes and lambs had ad libitum access to water and minerals, and were regularly treated with antihelminthics to minimize parasitic infection.

4.3.1 Prenatal and Postnatal treatments

Treatment groups included control (C) (n=6), prenatal rosiglitazone only (CR) (n=5), postnatal rosiglitazone only (C+R) (n=6), postnatal flutamide only (C+F) (n=5), prenatal testosterone (T) (n=5), prenatal testosterone and flutamide (TF) (n=9), prenatal testosterone and rosiglitazone (TR) (n=7), prenatal testosterone and postnatal rosiglitazone (T+R) (n=6). A prenatal testosterone and postnatal flutamide (T+F) group was omitted from the study due to uterine hypertrophy resulting in an inability to ovariectomize the animals prior to sacrifice. Prenatal T treatment involved twice weekly

i.m injections of T propionate (100 mg/2ml dissolved in cottonseed oil; Sigma-Aldrich, St. Louis, MO) suspended in corn oil from days 30 to 90 of gestation. The C group received similar volume of vehicle injections. Prenatal insulin sensitizer treatment involved daily oral treatment of insulin sensitizer at a dose of 8 mg/ewe. This dose of insulin sensitizer administered postpubertally improved reproductive function in prenatal T-treated females (*25*). Prenatal androgen antagonist treatment involved s.c injections of flutamide (15 mg/kg·d) as described earlier (*40*).

Postnatal treatments with insulin sensitizer began at 8 weeks of age and involved daily oral administration of the insulin sensitizer (0.11 mg/kg). The administered dose is comparable with that given to women with polycystic ovary syndrome (PCOS) (*41-42*). The same dose administered postpubertally was effective in improving reproductive cycle attributes of prenatal T treated females (*25*).

4.3.2 Tissue Collection

Brains were collected during the breeding season when females were 2 years of age. In order to normalize endogenous hormone levels among the groups, 3 to 4 weeks prior to tissue collection, ewes were ovariectomized and were treated with two controlled internal drug release P_4 implants (CIDR) (InterAG, Hamilton, Waikato, New Zealand) for 11–12 d and then for 1 day with four 3-cm-long E_2 implants to simulate ovarian steroid levels during the late follicular phase of the cycle. Ewes were sacrificed approximately 18 hours after E_2 implants. At time of tissue collection, all ewes received two intravenous injections (at 10 min intervals) of 25,000 U heparin (catalog # 402588B; Abraxiz Pharmaceuticals, Schumberry, IL). Ewes were anaesthetized intravenously with sodium

pentobarbital (390 mg/ml/kg; Fatal Plus, Vortech, Dearborn, MI) and rapidly decapitated. The heads were immediately perfused through the internal carotid artery with 6L of 4% paraformaldehyde (Sigma-Aldrich) dissolved in 0.1M phosphate buffer (PB; Sigma-Aldrich) containing 0.1% sodium nitrate (Sigma- Aldrich), and 10 U/ml heparin (Abraxiz Pharmaceuticals). Brains were submerged in the same fixative at 4°C for 18hr. Blocks of tissue containing hypothalami were transferred in 30% sucrose at 4°C and sectioned coronally (45 µm) using a freezing microtome (Microm, Walldorf, Germany) into 6 parallel series for each animal. Sectioned tissues were stored in cryoprotectant (30% ethylene glycol, 0.1% sodium azide, 30% sucrose in PB) at -20°C until further processing.

4.3.3 Immunohistochemistry: general

A series of every sixth section through the rostral-caudal extent of the ARC from each animal were processed for immunohistochemistry. All incubations occurred at room temperature with gentle agitation. Free-floating sections were rinsed thoroughly with 0.1 M phosphate-buffered saline (PBS) between incubations. Antibodies were dissolved in incubation solution consisting of 0.1M PBS, 0.4% Triton X-100 (catalog item BP151-500; Sigma-Aldrich) containing 4% normal goat serum (NGS) (catalog item 005-000-121; Jackson Immuno Research Laboratories, West Grove, PA). Unless otherwise specified, tissue sections were washed extensively with 0.1 M PBS (pH 7.35) between steps. Prior to incubation with the first primary antibody, sections were incubated with 1% hydrogen peroxide (10 min, H_2O_2; catalog item H325; Fisher Scientific, Pittsburgh,

PA) and incubation solution (1hr) to prevent non-specific background labeling. Negative controls were performed by omission of primary antibody, and resulted in elimination of all labeling corresponding to that antigen.

4.3.4 Immunohistochemistry: triple-label immunofluorescence for IR/AgRP/Kiss

Tissue sections were incubated overnight with primary antibody against the beta sub-unit of the insulin receptor (IRβ, raised in Rabbit, 1:300 dilution, Santa Cruz, C-19, SC-711), with biotinylated goat anti-rabbit (1:500 dilution, 1 hour, Vector Laboratories, Burlingame, CA, USA), ABC-elite (1:500 dilution in PBS, 1 hour, Vector Laboratories), biotinylated tyramide (BT; 1:250 dilution in PBS with 1 µl of 3% H_2O_2/ml, 10 minutes, Perkin Elmer Life Sciences, Woodbridge, Ont., Canada), and Alexa 555 conjugated streptavidin (1:100 dilution in PBS, 30 mins., Molecular Probes). Next, sections were incubated overnight (17 hours) with an antibody specifically recognizing AgRP (raised in Guinea Pig, 1:5000 dilution in incubation solution with 4% NGS; Antibodies Australia, catalogue # GPAAGRP.1, Lot # AS506), with biotinylated goat anti-Guinea Pig (1:500 dilution, 1 hour, Vector Laboratories, Burlingame, CA, USA), ABC-elite (1:500 dilution in PBS, 1 hour, Vector Laboratories), biotinylated tyramide (BT; 1:250 dilution in PBS with 1 µl of 3% H_2O_2/ml, 10 minutes, Perkin Elmer Life Sciences, Woodbridge, Ont., Canada), and Cy5 conjugated streptavidin (1:100 dilution in PBS, 30 mins., Molecular Probes). Finally, sections were incubated overnight (17 hours) with an antibody specifically recognizing kisspeptin (raised in rabbit, 1:200,000 dilution; kp10, lot 564;

gift from Dr. Alain Caraty, Nouzilly, France) and then with goat anti-rabbit Alexa 488 (1:100 dilution in PBS, 30 minutes, Molecular Probes). Sections were mounted on plus charged slides and cover slipped with gelvatol (*43*).

Specificity of the primary antibody for AGRP was previously been demonstrated in sheep hypothalamic sections (*43*). Pre-absorption of IRβ antibody with its corresponding peptide antigen (Santa Cruz) at concentration of 10 µg/ml for 24 hours at 4°C eliminated all immunoreactive staining (Supplemental Fig. 1). Finally, elimination of any single primary antibody while performing all other steps of the protocol eliminated all staining for the corresponding antigen and no others, demonstrating lack of cross-reactivity.

4.3.5 Immunohistochemistry: dual-label immunoperoxidase for AR/kisspeptin and AR/AgRP

Tissue sections were incubated with rabbit anti-AR (1: 200 dilution, 17 h; Santa Cruz, sc-816, lot F1711), biotinylated goat anti-rabbit IgG (1:500, 1 hr; catalog item BA-9200; Vector Laboratories, Burlingame, CA), avidin-biotin horseradish peroxidase complex (ABC; 1:500, 1 hr, catalog item PK-6100; Vector Laboratories), and 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Alderich) with 0.08% nickel sulfate (10 min; diluted in PB containing 0.012% H_2O_2). Next, sections were incubated overnight (17h) with rabbit anti-kisspeptin (1:300,000 dilution; kp10, lot 564; gift from Dr. Alain Caraty, Nouzilly, France) or guinea pig anti-AgRP (1:1000 dilution, C-GPAAGRP, Lot AS-506, Antibodies Australia), biotinylated goat anti-rabbit IgG (1:500,

1 hr; catalog item BA-9200; Vector Laboratories, Burlingame, CA), ABC (1:500, 1 hr, catalog item PK-6100; Vector Laboratories), and 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Alderich,10 min; diluted in PB containing 0.012% H_2O_2). Tissue sections were mounted onto Superfrost/Plus Microscope Slides (Fisher Scientific), dehydrated with alcohol and cover-slipped with Depex Mountant (Electron Microscopy Sciences, Fort Washington, PA).

4.3.6 Analysis

Colocalization of AR/kiss and AR/AgRP: All kisspeptin- and AgRP-positive neurons, in 3-4 hemisections through the middle ARC for each animal, were analyzed for colocalization of AR using a LeicaDM5000B at 20x magnification. The middle ARC was defined as that rostral-caudal level of the nucleus directly adjacent to the tubero-infundibular sulcus of the third ventricle. Co-localized neurons were defined by the presence of a blue-black AR-positive nucleus completely surrounded by brown kisspeptin or AgRP-positive cytoplasm, as examined in the same plane of focus. The percentages of kisspeptin and AgRP neurons that colocalized AR were calculated for each section and averaged per animal. Comparisons between treatment groups were conducted using ANOVA, and Dunn's or Holm-Sidak post-hoc test, with 95% confidence levels.

Colocalization of IR/kiss and IR/AgRP: Fluorescent images spanning the entire middle ARC in 3-4 hemi-sections per animal were captured using a cooled CCD camera (Microfire, Optronics) attached to a Leica microscope (DM5000B, Leica Microsystems;

Wetzlar, Germany) and Neurolucida software (MicroBrightfield Inc.) with fixed camera settings for all subjects (using 20x objectives). The number of single-labeled kisspeptin and AgRP neurons, and double-labeled IR/kisspeptin and IR/AgRP neurons, were manually counted using Neurolucida software (MBF Bioscience, Williston, VT) and the percentage co-localization calculated in each animal. Group means of percent colocalization of IR/kisspeptin or IR/AgRP were compared using ANOVA and Dunn's or Holm-Sidak, with 95% confidence levels.

4.4 Results

4.4.1 AgRP neuronal numbers

In agreement with previous finding (*33*) prenatal T treated females had significantly more arcuate AgRP neurons compared to control females ($F(6,43):22.36$; $p<0.001$) and TF treatment was able to reverse this effect ($p<0.001$) (Fig. 20). Prenatal (TR), but not postnatal Rosiglitazone treatment (T+R) was also able to reverse the effects of prenatal T on AgRP neuronal numbers in the ARC ($p<0.001$). There were no significant differences between any of the control groups (C, C+F, C+R, and CR).

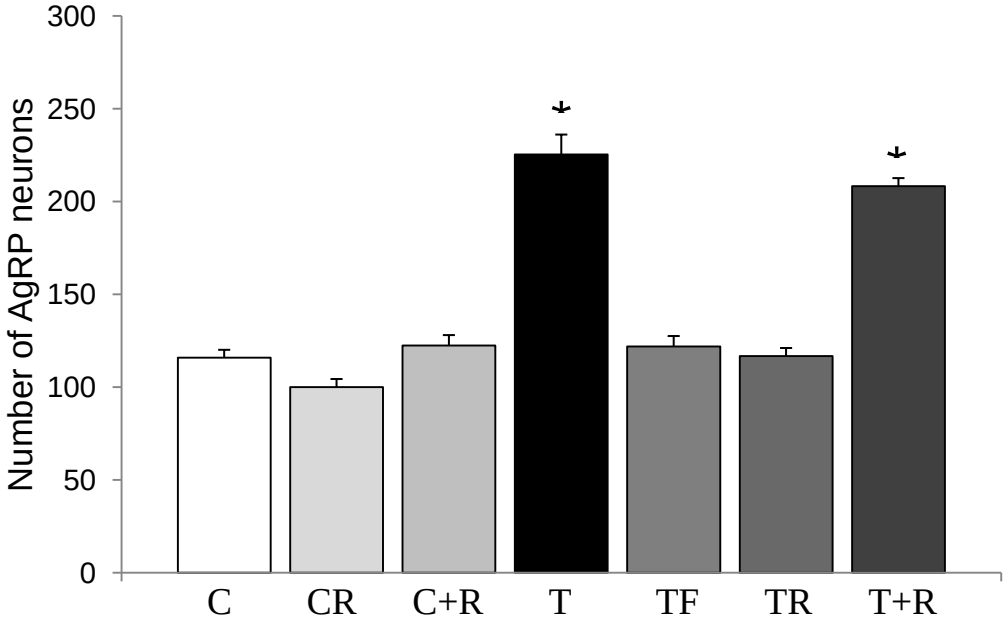

Figure 19. Mean ± SEM number of arcuate AgRP neurons in control (C), prenatal rosiglitazone only (CR), postnatal rosiglitazone only (C+R), prenatal testosterone (T), prenatal testosterone and prenatal flutamide (TF), prenatal testosterone and prenatal rosiglitazone (TR), and prenatal testosterone and postnatal rosiglitazone (T+R) treated females. * describes significant difference from controls.

4.4.2 Androgen receptor colocalization with KNDy and AgRP neurons

As shown previously (*35*), prenatal T treated females exhibited an increased percentage of AR colocalization within arcuate kisspeptin (KNDy) neurons ($F(7,36):15.93$; $p<0.001$) and AgRP neurons ($F(7,37):27.02$; $p<0.001$) and both effects were reversed by prenatal co-treatment with androgen blocker, Flutamide (TF) ($p<0.001$) (Fig. 18; Table 2). By contrast, prenatal or postnatal treatment with the insulin sensitizer, Rosiglitazone (TR) failed to reverse the effects of prenatal T on AR colocalization within KNDy or AgRP neurons, and these groups were not significantly different from controls.

4.4.3 Insulin receptor colocalization with KNDy and AgRP neurons

Again, our previous findings (*36*) were replicated in that prenatal T treated females had decreased IR colocalization within both KNDy ($F(7,38):24.92$; $p<0.001$) and AgRP ($F(7,39):8.98$; $p<0.001$) neurons (Fig. 19; Table 2). Prenatal co-treatment with androgen blocker, Flutamide (TF) reversed the effect of prenatal T on IR colocalization within AgRP ($p<0.001$), but not IR colocalization in KNDy neurons. Prenatal, but not postnatal ($p=0.269$) treatment with the insulin sensitizer, Rosiglitazone (TR) reversed the effects of prenatal T on IR colocalization within both KNDy ($p<0.001$) and AgRP ($p<0.001$) neurons.

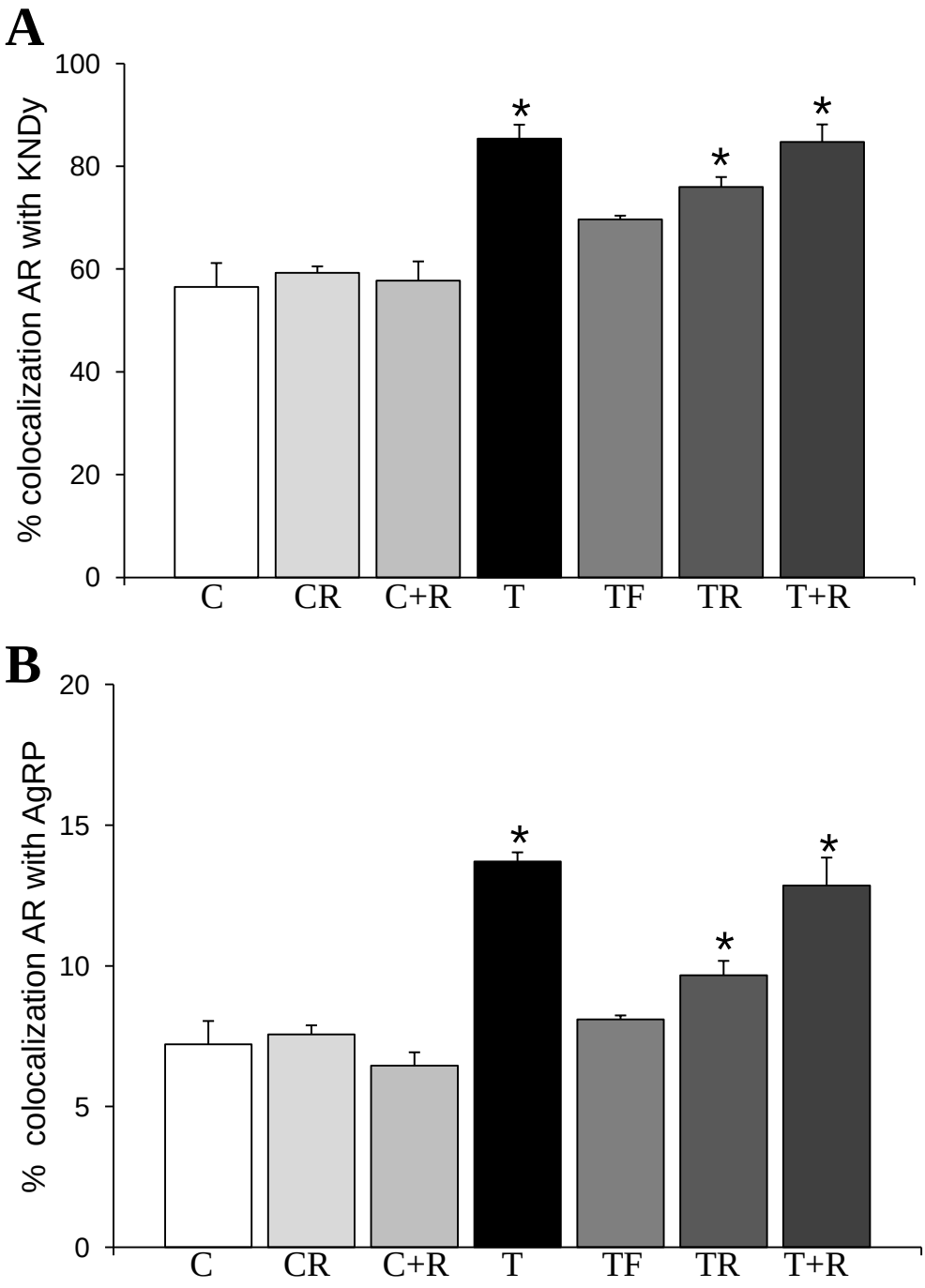

Figure 20. Mean ± SEM percent colocalization of androgen receptor (AR) with arcuate KNDy (A) and AgRP (B) neurons in control (C), prenatal rosiglitazone only (CR), postnatal rosiglitazone only (C+R), prenatal testosterone (T), prenatal testosterone and prenatal flutamide (TF), prenatal testosterone and prenatal rosiglitazone (TR), and prenatal testosterone and postnatal rosiglitazone (T+R) treated females. * describes significant difference from controls.

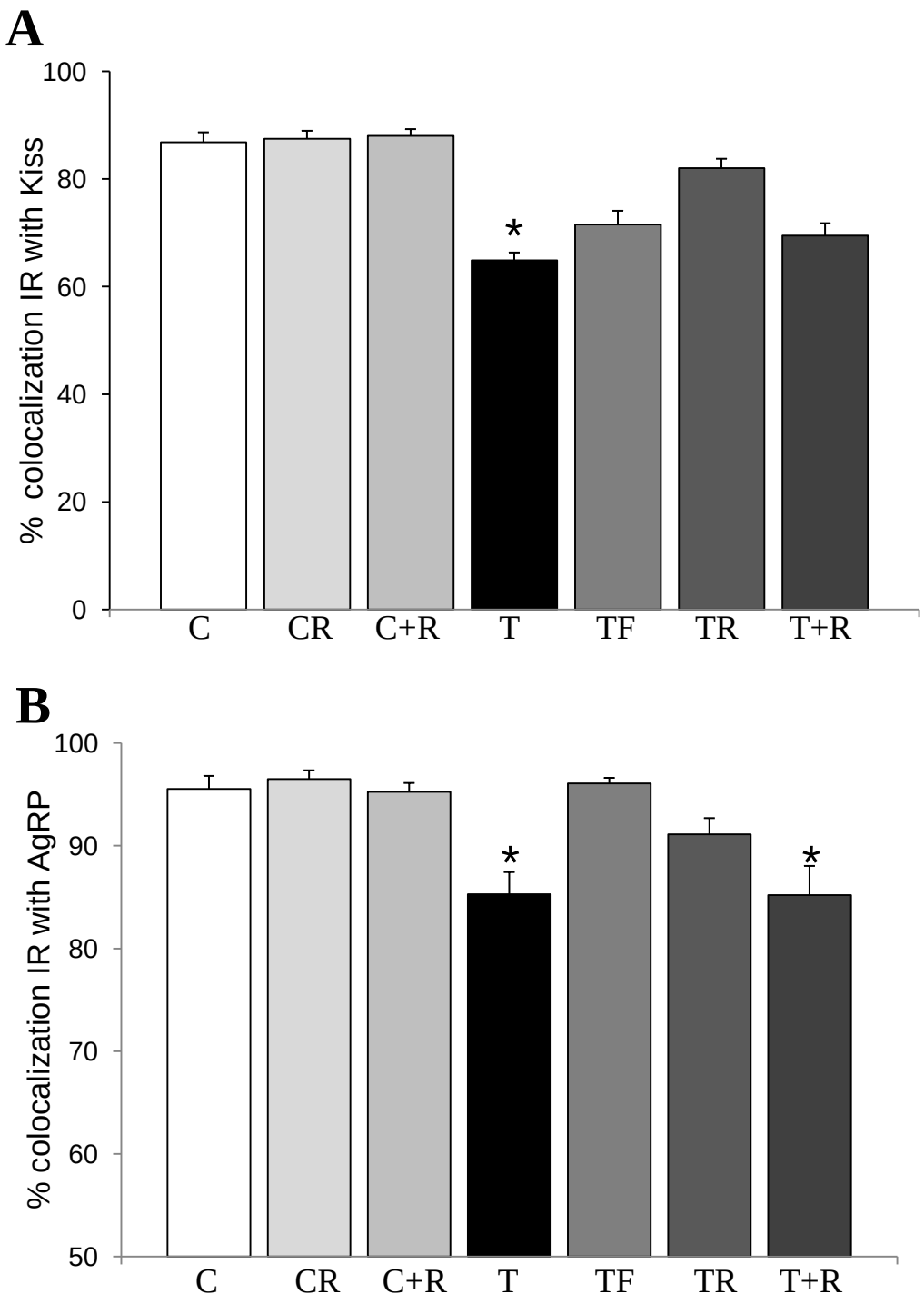

Figure 21. Mean ± SEM percent colocalization of insulin receptor (IR) with arcuate KNDy (A) and AgRP (B) neurons in control (C), prenatal rosiglitazone only (CR), postnatal rosiglitazone only (C+R), prenatal testosterone (T), prenatal testosterone and prenatal flutamide (TF), prenatal testosterone and prenatal rosiglitazone (TR), and prenatal testosterone and postnatal rosiglitazone (T+R) treated females. * describes significant difference from controls.

	Prenatal Intervention, androgen blocker (F)	Prenatal Intervention, insulin sensitizer (R)	Postnatal Intervention, insulin sensitizer (R)
AR/KNDy	√	X	X
AR/AgRP	√	X	X
IR/KNDy	X	√	X
IR/AGRP	√	√	X

Table 2. Effects of pre- and postnatal treatment with the androgen blocker, Flutamide (F) and the insulin sensitizer, Rosiglitazone (R) on prenatal T induced effects on androgen receptor (AR) and insulin receptor (IR) colocalization with the arcuate KNDy and AgRP neurons

4.5 Conclusions

The findings in this study show that prenatal, but not postnatal interventions increasing insulin sensitivity with Rosiglitazone were able to prevent the effects of prenatal T treatment on insulin receptor (IR) and androgen receptor (AR) colocalization with ARC KNDy and AgRP neurons as well as on AgRP neuronal numbers. Thus the effects of prenatal T on AgRP peptide, as well as colocalization of IR and AR in both AgRP and KNDy neurons, depends on insulin signaling. This points to a convergence of both signals, T and insulin, at the level of the fetal brain to produce long-term changes in hypothalamic neuropeptide/receptor expression (Fig. 22).

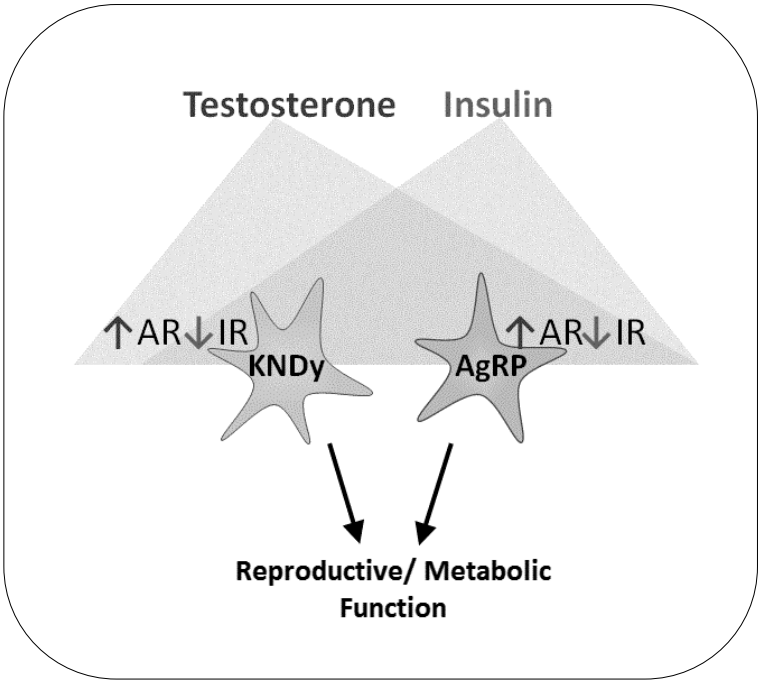

Figure 22. Schematic diagram illustrating the effects of prenatal exposure to excess testosterone and insulin on androgen receptor (AR) and insulin receptor (IR) expression in arcuate (ARC) KNDy and AgRP neurons and the potential impact on reproductive and metabolic function.

4.5.1 Prenatal Interventions

We replicated previous findings from our lab showing that prenatal T-treated ewes exhibit an increase in the number of AgRP (appetite stimulating), but not POMC (appetite suppressing) neurons in the ARC, an effect which is reversed by prenatal co-treatment with Flutamide (TF) (*33*). We also replicated findings that prenatal T-treated ewes show a decrease in IR and increase in AR colocalization within AgRP and KNDy neurons (*36*). Each of these effects were blocked by co-treatment with androgen blocker (TF), with the exception of IR colocalization with KNDy neurons, suggesting that the majority of these alterations are programmed by a mechanism requiring the androgenic actions of T. This is consistent with our previous findings showing that prenatal dihydrotestosterone (DHT) treatment mimics the effects of T, while prenatal Estrogen (E) treatment has no effect on AR-ir (*35*). By contrast, the effect of prenatal T in decreasing colocalization of IR in KNDy neurons appears to depend on the estrogenic actions of the T treatment, presumably via aromatization of T. The estrogenic nature of this effect, however, needs to be confirmed by either prenatal E treatment, or by the ability of aromatase inhibitors to block it.

The ability of prenatal co-treatment with insulin sensitizer (TR) to block the effects of T on IR colocalization with AgRP and KNDy neurons as well as AgRP neuronal numbers suggests that IR expression in these neurons is organized by a mechanism requiring insulin signalling. Because prenatal treatments with either insulin-sensitizer or anti-androgen can block the effects of prenatal T on the AgRP population (IR colocalization

and neuronal number), this indicates that a common mediator involving both androgen and insulin signaling is responsible for the long-term alterations in this population. Increases in circulating insulin levels in the mother associated with T treatment (Abi Salloum, B., and V. Padmanabhan, unpublished data) may provide a means by which insulin mediates some organizational programming in the developing fetus. It is also conceivable that exposure of the fetus to high levels of T as well as insulin may program certain aspects of the reproductive and metabolic system via a pathway that involves both influences. For example, it is well documented that hyperinsulinemia exacerbates hyperandrogenemia by acting synergistically with amplified LH levels to increase androgen production in theca cells of the ovary (*44-47*). Moreover, insulin suppress sex hormone binding globulin (SHBG) produced by the liver, permitting an increase in unbound plasma testosterone (*48-49*). Thus, increased insulin levels in the pregnant ewe may intensify the effects of T on the fetus, while acting in parallel to program aspects of the prenatal T phenotype.

Prenatal co-treatment with insulin sensitizer (R) blocked the effects of T on IR colocalization with KNDy neurons, an effect that was not achieved with by prenatal co-treatment with the anti-androgen, Flutamide. As noted above, this suggests that the effects of prenatal T on IR in this population are programmed via a mechanism involving insulin signaling and perhaps the estrogenic actions of T. Indeed, fetuses exposed to T show an increase in circulating estrogen (*50*), providing a means for such estrogenic mediation. Previous studies have shown that prenatal T, acting via an androgenic

pathway, programs estradiol-negative feedback disruptions in the sheep, while disruptions in the GnRH/LH surge are programmed by the estrogenic actions of T (*51*). Because IR expression in KNDy neurons may be programmed via a mechanism involving estrogen and insulin signalling, it is tempting to speculate that restoring IR expression in KNDy neurons may partially restore the GnRH/LH surge. However, this does not appear to be the case as prenatal co-treatment with R does not reverse the effects of prenatal T treatment on the GnRH/LH surge (*26*). Moreover, a recent study in mice showed selective IR deletions from ARC kisspeptin (KNDy) neurons caused a delay in the onset of puberty, but fertility was not affected (*52*). These studies suggest that IR expression in KNDy neurons is not essential in the control of GnRH secretion, but may play an important role in the initiation of puberty. Although our group of females (ovary intact up to 2 years of age, then OVX) do not display alterations in the onset of puberty (*16*), prenatal T treated sheep that have been OVX and E-replaced shortly after birth display advanced puberty onset (*53*). It may be that prenatal T induced decrease in IR expression within KNDy neurons contributes to the timing of puberty onset, but in our model additional ovarian factors counter the functional effect of the reduction in IR (*16*). Nevertheless, it would be worthwhile to compare the effects of prenatal T treatment on IR/KNDy colocalization on the onset of puberty between these two sheep models (OVX+E replaced and ovary intact).

AgRP expression is regulated by insulin levels, with increased insulin levels suppressing AgRP and vice versa (*54-56*). Prenatal treatment with insulin sensitizer blocked the

prenatal T induced reduction of IR colocalization with AgRP. As such, it is tempting to assume that by normalizing IR signalling in this population, insulin can appropriately regulate AgRP, decreasing its expressing to levels comparable to control females. As such, blocking the effects of high maternal insulin on the developing fetus may decrease the probability for insulin resistance and improve metabolic function in the adult offspring.

4.5.2 Postnatal treatments

Failure of postnatal treatment with insulin sensitizer to reverse the effects of prenatal T on AR and IR colocalization within KNDY and AgRP neurons, as well as its effect on number of AgRP neurons, suggests that these components are programmed during the prenatal period. Insulin sensitizers are commonly used in the treatment of hyperinsulinemia and hyperandrogenemia in adolescent or adult women with PCOS (*7, 57-59*). Given the close similarities between the metabolic and reproductive dysfunctions seen in prenatal T sheep and women with PCOS (ref), it was disappointing that postnatal treatment with rosiglitazone did not reverse the effects of prenatal T-treatment on the KNDy and AgRP populations in the sheep model. It may be that the organization of IR and AR expression in these populations is important during the early stages of development in order to set the framework for dysfunctions later in life. In this regard, it is interesting that although interventions with androgen antagonist (*60*), insulin sensitizer (*61-63*), or combined (*41, 64*) are able to improve ovulation rate in subjects with PCOS, optimal success with pregnancy rate are yet to be realized. It is possible that postnatal

interventions are able to alleviate some dysfunctions associated with PCOS; however, in cases where prenatal origins of this disease are a factor, it may be that some neuroendocrine mechanisms remain permanently altered by prenatal T treatment and alter the threshold for complete restoration of successful reproductive and metabolic function.

Although we saw no effect of postnatal insulin sensitizer treatments on androgen or insulin receptor expression, or AgRP expression, this does not exclude an effect on other neuropeptide expression. Preliminary data from our lab indicates that postnatal treatment with insulin sensitizer reverses the effects of prenatal T treatment on some but not all KNDy neuropeptide expression, increasing numbers of Neurokinin B (NKB) neurons to levels comparable to controls but having no effect on the number of dynorphin neurons *(Lehman, M.N., preliminary data).* Therefore, it may be that different neuropeptide/receptor components of the KNDY are programmed during different critical periods, with AR and IR being programmed primarily during the prenatal period and setting the framework for later programming of NKB expression, which may be reversed by treatments during the postnatal period. It is particularly interesting to note that the three KNDy peptides, all co-expressed a single set of neurons, are each regulated by different critical periods and signals: kisspeptin, whose expression is independent of prenatal T; NKB which is decreased by prenatal T and dependent on both pre- and post-natal insulin signaling; and dynorphin, which is decreased by prenatal T and dependent on only prenatal insulin action. Given the role of ovine KNDy neurons in both pulsatile and surge secretion of GnRH, it is likely that the functional impact of this differential

programming is likely seen at the level mechanisms controlling puberty, as well as adult estrous cyclicity, but the precise functional roles remain to be determined.

4.5.3 Conclusions and clinical implications

Due to the adverse side effects of the anti-androgens and the risk of feminization of a male fetus (*65*), the use of anti-androgens are contraindicated during pregnancy. However, insulin sensitizers, such as Rosiglitazone have been widely used in pregnant women with PCOS to reduce gestational complications such as miscarriage, gestational diabetes and pre-eclampsia in the mother (*66-67*). However, studies examining the effects of prenatal treatment with insulin sensitizers on offspring, during infancy and adult life, are limited. *Carlsen* and *Vanky (2009)* showed that newborns of mothers with PCOS treated with the insulin sensitizer Metformin during gestation had higher levels of SHBG, which is suppressed by hyperinsulinemia (*49*), but no change in androgen or estrogen levels (*68-69*), suggesting a role for insulin sensitizer in improving gestation hyperinsulinemia. However, due to the shortage of studies examining the effects of prenatal exposure to insulin sensitizers during adulthood, we cannot conclude whether this intervention influences testosterone levels or improves metabolic and reproductive functions later in life.

The findings from this study provide evidence in the support of prenatal programming of receptors that are involved in insulin and androgen signaling within reproductive and metabolic neurons of the hypothalamus, the KNDy and AgRP neuronal populations,

respectively. The programming of these receptors provides a framework though which hyperandrogenemia and hyperinsulinemia are able to exert their effects on these neuronal populations. The ability of prenatal, but not postnatal intervention with insulin sensitizer to block the effects of prenatal T treatment on the IR and AR colocalization within KNDy and AgRP neurons suggests that the prenatal period may provide a critical time point to intervene and prevent long-term changes in hypothalamic peptide/receptor expression, which otherwise may remain permanently modified by prenatal T. This study is among the first to show that prenatal interventions blocking fetal exposure to excess androgen or insulin levels may prevent some fundamental disturbances in receptor balance which, in turn, may subsequently alter the threshold required for normal metabolic and reproductive functions. In this way, the changes we identified may represent a potential neuroendocrine basis for the effectiveness of therapies that are already being used in the treatment of women with PCOS.

Chapter 4: References

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Chapter 5: Neural pathways by which leptin may modulate activity of kisspeptin (KNDy) neurons in the sheep: activation of AgRP and POMC neurons.

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5.1 Abstract

Leptin serves as an indicator of peripheral energy stores and a permissive metabolic signal for reproductive function and the initiation of puberty. Leptin modulates pulsatile gonadotropin secretion from the anterior pituitary via the regulation of hypothalamic gonadotropin releasing hormone (GnRH) neurons. The arcuate (ARC) KNDy neurons (co-expressing the neuropeptides kisspeptin, neurokinin B/dynorphin) are important neurons involved in the control of pulsatile GnRH secretion and may comprise one of the pathways by which leptin influences reproduction. Neuronal activation by leptin can be studied by examining phosphorylation of STAT3 (pSTAT3), a direct consequence of the binding of leptin to its receptor. In addition, induction of the immediate early gene product, cFos can be used as an indicator of general activation. We examined leptin-induced pSTAT3 and cFos in sheep brain to delineate the neural pathways by which leptin might regulate GnRH secretion in this species. Adult, ovary-intact ewes received injections of either leptin (1mg/kg, i.v.) or vehicle, and two hours later were euthanized and their brains perfused for immunocytochemistry. Alternate series of sections were processed for dual immunoperoxidase detection of pSTAT3 or cFos with either kisspeptin, agouti-related peptide (AgRP), proopiomelanocortin (POMC), or GnRH. Leptin-treated sheep showed pSTAT3 expression in a majority of AgRP neurons (84%) and a subset of POMC neurons (30%), but not in kisspeptin or GnRH neurons; control, vehicle-injected animals exhibited no pSTAT3 in any of these neurons. By contrast, leptin induced cFos expression in a majority of ARC kisspeptin (70%) and POMC (92%)

neurons, while control animals showed 20-30% basal levels of co-expression. AgRP neurons showed high levels expression of cFos in both leptin and control animals (70-90%). GnRH neurons were not examined for cFos expression. In summary, leptin appears to directly activate AgRP and POMC, but not kisspeptin and GnRH, neurons by activation of leptin receptors (LepR) and STAT3. Moreover, ARC kisspeptin neurons are also activated by leptin but indirectly, presumably by afferents from other neurons that in turn are leptin-responsive. These results suggest that leptin may modulate GnRH secretion in sheep by a pathway that starts with AgRP and POMC neurons, but may also include projections to KNDy neurons and their inputs to GnRH neurons.

5.2 Introduction

Leptin is an important metabolic signal of energy stores and an indicator to the reproductive axis whether sufficient energy is available to initiate puberty, permit reproductive activity and maintain reproductive functions (*1-5*). High leptin concentrations, often linked with obesity or polycystic ovarian syndrome (PCOS), can partially explain the negative impact of these pathologies on fertility (*6-7*). Although leptin can regulate reproduction via actions that ultimately control the secretion of hypothalamic gonadotropin releasing hormone (GnRH) , this influence is indirect (8) and the exact mechanisms through which leptin affects GnRH neuronal activity remain unclear. Animal models can provide a means of studying the signaling pathways of leptin in the brain. Leptin-deficient ($Lep^{ob/ob}$) mice are infertile (*8*) and this is most likely due reduced circulating gonadal steroids (*9*) and decreased sensitivity of the hypothalamic-pituitary system to steroidal signals (*10*). Reversing leptin deficiency in these mice by peripheral leptin injections, stimulates the reproductive axis, reverses the hypogonadotropic hypogonadism and restores fertility (*11-14*). A wide variety of studies have shown that leptin activates the neuroendocrine reproductive axis in many species including rodents (*12, 15-16*) and primates (*17*) by stimulating GnRH and LH secretion. However, dual-labeling studies using in-situ hybridization and immunohistochemistry have shown few, if any GnRH neurons expressing leptin receptors (LepR) in rats (*18*) and monkeys (*17*). While this does not dismiss the probability that leptin can act directly on GnRH neurons, a more likely mechanism may involve indirect influence via interneurons upstream of GnRH (*19*).

The ARC KNDy neurons (expressing the neuropeptides, kisspeptin, dynorphin and neurokinin B) have emerged as a key population of neurons involved in relaying the feedback influence of gonadal hormones onto GnRH neurons (*20-22*). GnRH is directly stimulated by kisspeptin and GnRH neurons express the kisspeptin receptor, *Kiss1R* (*23-26*). Furthermore, mutations of *Kiss1R* result in reproductive dysfunction such as delayed or absent pubertal maturation, low circulating gonadotropin levels, and hypogonadotropic hypogonadism in humans (*27*) and mice (*25, 28-31*). Kisspeptin is also influenced by nutritional status and circulating leptin levels. $Lep^{ob/ob}$ mice show decreased Kiss1 expression, while central administration of leptin increases kisspeptin mRNA (*32-33*). Although the expression of LepR in kisspeptin neurons has been a topic of debate, recent work has shown that very few ARC KNDy neurons express LepRb and directly respond to leptin (by activation of signal transducer and activator of transcription (STAT) 3)(*34*), suggesting that leptin's influence on kisspeptin may be indirect via inputs from afferent leptin-sensitive neurons. The appetite stimulatory and inhibitory neurons of the ARC, agouti-related peptide (AgRP) and proopiomelanocortin (POMC) neurons, respectively, are key targets of leptin as they express high affinity leptin receptors (*18, 35*). Moreover, direct synaptic connections exist between the ARC KNDy neurons and AgRP and POMC neurons (*36-37*). In this study, we determined which neuronal populations in the sheep hypothalamus are directly activated by leptin by examining phosphorylation of STAT3 (pSTAT3) expression, a direct consequence of the binding of leptin to its receptor. In addition, we examined induction of the immediate early gene product, cFos, as a general marker of neuronal activation in the same animals. In conjuncture with our previous

findings (*38*), our goal was to characterize direct and indirect leptin signalling in the ewe brain within neurons implicated in metabolism and reproduction, and by doing so delineate the neural pathways by which leptin may regulate GnRH secretion in this species.

5.3 Materials and Methods

5.3.1 Animals and experimental groups

Adult Suffolk ewes (2.5-3 years of age) were cared for at the University of Michigan Sheep Research Facility (Ann Arbor, MI). Details of housing and nutrition have previously been described (*39-41*). For this experiment, the animals were ovary intact and sacrificed during the anestrous season. Twenty-four hours before sacrifice animals were placed off-feed. Two hours prior to sacrifice, sheep were administered peripheral injections (i.v.) of leptin (0.8ml/mg in 15mM HCl and 7.5mM NaOH (pH 5.2) human recombinant leptin, Sigma-Alderich catalogue # L4146; n=4). Control ewes received i.v. injection of vehicle (0.5 ml HCl and 0.3 ml NaOH; n=1) or no injection (n=3). To examine the effects of short-term food restriction on neuronal activity in the brain, an additional control group of animals was added, which was provided ad-libitum food up to the time of sacrifice.

5.3.2 Tissue Collection

At time of tissue collection, all ewes received two intravenous injections (at 10 min intervals) of 25,000 U heparin (Abraxiz Pharmaceuticals). Ewes were heavily anaesthetized intravenously with sodium pentobarbital (390 mg/ml/kg; Fatal Plus, Vortech, Dearborn, MI) and killed by rapid decapitation. Ewe heads were perfused through the internal carotid artery with 6L 4% paraformaldehyde (Sigma-Alderich) dissolved in 0.1M sodium phosphate buffer (PB; Sigma-Alderich) containing 0.1% sodium nitrate (Sigma-Alderich). Brains were submerged in the same fixative at 4 degrees C for 18hr. Blocks of tissue containing hypothalami were then kept in 30%

sucrose (Caledon) at 4°C and sectioned coronally using a freezing microtome (Microm, Walldorf, Germany) into 12 parallel series (45 □m) for each animal. Sectioned tissues were stored in cryoprotectant (30% ethylene glycol, 0.1% sodium azide, 30% sucrose in PB) at -20°C until further processing.

5.3.3 Dual-label immunoperoxidase staining: GnRH, Kisspeptin, POMC, or AgRP with pSTAT3 or cFos

Series of every six section through the hypothalamus of each animal were processed using immunohistochemistry. All incubations occurred at room temperature with gentle agitation. Free floating sections were rinsed thoroughly with 0.1 M sodium phosphate-buffered saline (PBS) between incubations. Tissue was incubated in 100% methanol (10 min; Caledon) and 30% H_2O_2 (10 min; Fisher Scientific), then blocked for an hour in incubation solution (PBS containing 0.4% Triton-X (Fisher Scientific) containing 4% normal goat serum (NGS, Jackson ImmunoResearch, West Grove, PA, USA). Sections were incubated overnight (17 hours) with antibody specifically recognizing pSTAT3 (raised in rabbit, 1:100 in incubation solution; Cell Signaling, Danvers, MA) or cFos (raised in rabbit, 1:1000, in incubation solution, Santa Cruz SC-52), with biotinylated goat anti-rabbit (1:250 in incubation solution with 4% NGS, 1 hour, Vector Laboratories), ABC-elite (1:250 in PBS, 1 hour, Vector Laboratories). Nuclear pSTAT3 staining was visualized with 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Alderich) with 0.08% nickel sulfate (10 min; diluted in PB containing 0.012% H_2O_2). Finally, sections were incubated overnight with antibody specifically recognizing POMC (raised in rabbit, 1:120,000 in incubation solution with 4% NGS, Phoenix

Pharmaceuticals, catalogue # H-029-30), AgRP (raised in guinea pig, 1:5000, incubation solution with 4% NGS, Antibodies Australia, C-GPAAGRP), kisspeptin (raised in rabbit, 1:100,000 in MBH, 1:20,000 in POA, in incubation solution with 4% NGS, gift from Dr. Alan Caraty, France, #564), or GnRH (raised in mouse, 1:2000, in incubation solution with 4% NGS, Covance, city, state). Next, sections were incubated with biotinylated goat anti-rabbit (for POMC and Kisspeptin; 1:500 in incubation solution with 4% NGS, 1 hour, Vector Laboratories) or biotinylated goat anti-rabbit (for GnRH; 1:300 in incubation solution with 4% NGS, 1 hour, Vector Laboratories), with ABC-elite (1:500 in PBS, 1 hour, Vector) and with DAB to visualize reaction product (10 minutes, 0.02% diluted in PB containing 0.012% H_2O_2). Tissue sections were mounted onto Superfrost/Plus Microscope Slides (Fisher Scientific), dehydrated with alcohol and cover-slipped with Depex Mountant (Electron Microscopy Sciences, Fort Washington, PA). Specificity of the primary antibodies for POMC, AgRP, kisspeptin, and GnRH has previously been demonstrated in sheep hypothalamic sections using preabsorption and omissions controls (*42-44*). Specificity of the pSTAT3 labeling was confirmed by preabsorption of the diluted antiserum with pSTAT3 immunizing peptide (Cell Signaling, catalogue # 1195) at concentration of 10μg/mL, for 24 h at 4° C; this procedure eliminated all labeling (Fig. A.2).

5.3.4 Triple-label fluorescence staining: Kisspeptin, AgRP and POMC

To confirm close contacts between kisspeptin, AgRP and POMC, triple label immunofluorescence was conducted on 3-5 tissue sections from the middle ARC. Briefly, to detect AgRP, tissue sections were incubated in guinea pig anti-AgRP serum (1:750;

Antibodies Australia, C-GPAAGRP; Lot-AS506) for 17 hr. AgRP was visualized with goat anti-guinea pig Alexa 488 (1:100; 30 min; Invitrogen, Carlsbad, CA). Next, tissue sections were incubated with polyclonal rabbit anti-kisspeptin-10 serum (1:200,000; gift from A. Caraty) for 17 hr. Next, kisspeptin was visualized by incubating sections with goat anti-rabbit (1:250; 1hr., Vector laboratories, Burlingame, CA), ABC (1:500; 1hr., Vector Laboratories, Burlingame, CA), biotinylated tyramide (TSA; 1:250; 10 min., diluted in PBS with 1 μl of 3% H2O2 per ml of total volume; PerkinElmer Life Sciences, Cat # NEL700A), and Alexa 555-conjugated streptavidin (1:100; 30 min; Jackson Immunoresearch Laboratories). Next, POMC was detected using rabbit anti-POMC (1:4000, catalog # H-029-30, Phoenix Pharmaceuticals, Burlingame, CA) for 17 hr. To visualize POMC, sections were incubated with donkey anti-rabbit Cy5 (1:100; 30 min; Jackson Immunoresearch Laboratories). Sections were mounted on gelatinized slides, dried and coverslipped with gelvatol. Specificity and validation of these antibodies in sheep tissues has been previously described (12, 44). Additional controls included omission of one of the primary antibodies from the protocol; this resulted in complete elimination of labelling for the corresponding antigen without any effect on the others.

5.3.5 Analysis

3-5 hemi-sections from each area of interest were quantitatively analyzed for colocalization of AgRP, POMC, kisspeptin and GnRH with pSTAT3 and cFos in leptin treated and control females. Single- and dual-labelled neurons were counted in the ARC

(AgRP, POMC, kisspeptin), POA (kisspeptin, GnRH), MBH (GnRH) and AHA (GnRH), and percent co-localization was determined.

5.3.6 Quantitative analysis of pSTAT3 and cFos colocalization within GnRH neurons

All GnRH neurons in POA, lateral hypothalamus (LH), anterior hypothalamic area (AHA), and MBH were analyzed for colocalization of pSTAT3 (already published (*38*)) and cFos using a LeicaDM5000B at 10-20x magnification. Percentages of GnRH neurons that co-expressed pSTAT3 and cFos were calculated for each brain region for each animal. Double-labeled neurons were defined as those in which a pSTAT or cFos-positive nucleus was seen within an immunoreactive GnRH neuronal body, both viewed under the same plane of focus. Comparisons between leptin-injected and control groups were conducted using ANOVA with 95% confidence levels.

5.3.7 Quantification of pSTAT3 and cFos with kisspeptin, POMC and AgRP

For each animal (control n=3; leptin n=3), the numbers of kisspeptin, POMC, and AgRP-ir neurons colocalizing pSTAT3 and cFos were counted in 3-5 hemi-sections taken from each of the rostral, middle, and caudal levels of the ARC, and in 3-5 sections through the POA, using Neurolucida software (Microbrightfield Inc., Williston, VT) attached to a motorized Leica DM5000B (Leica Microsystems,Wetzlar, Germany) microscope and Leica DFC420 camera. As for GnRH neurons, dual-labeled neurons were defined as those in which a pSTAT or cFos-positive nucleus was seen completely surrounded by kisspeptin-, POMC-, or AgRP-immunoreactive cytoplasm, when viewed under the same plane of focus. Percentages of kisspeptin, POMC, or AgRP neurons colocalizing pSTAT3

and cFos were calculated for each animal per brain area and/or per rostral-caudal level. In the ARC, effects of treatment and rostral-caudal level were analyzed using two-way ANOVA. In POA, treatment effects were analyzed using t-tests.

5.3.8 Images

All images were captured using Neurolucida software (Microbrightfield Inc.) attached to a motorized Leica DM5000B (Leica Microsystems) microscope and Leica DFC420 camera. Figures were prepared using Adobe Photoshop CS2. Images were only adjusted for brightness and contrast, and not altered in any other way.

5.4 Results

5.4.1 pSTAT3 colocalization within AgRP, POMC, kisspeptin and GnRH neurons

Leptin-treated sheep showed pSTAT3 expression in a majority of AgRP (84% ±2.17) and POMC (30% ±2.33) neuron, By contrast, we failed to detect any pSTAT3 out of over 1000 kisspeptin neurons examined in the ARC and POA, and similarly failed to see pSTAT3 in over 500 GnRH neurons examined, regardless of their location in the POA, AHA or MBH. In addition, control and vehicle-injected animals were devoid of pSTAT3 staining in any hypothalamic region.

5.4.2 Regional differences in pSTAT3 colocalization within POMC and AgRP neurons

Within the ARC, there were significant differences in the percentage of pSTAT3/POMC colocalization depending on rostral-caudal level of the nucleus ($F(2,17; p = 0.004)$. Specifically, we saw a higher degree of colocalization of pSTAT3/POMC in the rostral and middle ARC compared to the caudal ARC in leptin injected animals ($p < 0.001$ and 0.002, respectively). By contrast, there were no significant regional differences among rostral-caudal levels of the ARC in percentage of pSTAT3/AgRP colocalization ($p=0.675$). In addition, there were no differences in number of POMC-ir or AgRP-ir neurons between leptin and control groups.

5.4.3 cFos colocalization within POMC, AgRP, and Kisspeptin Neurons

Leptin induced cFos colocalization (e.g., Figs. 24 and 25) in a majority of ARC kisspeptin (70% ±6.68), POMC (92% ±1.82) and AgRP (88% ±0.72) neurons

(F(2,9):41.63-73.21, $p<0.001$), while fed control animals showed 1.6% ±0.78, 13.62% ±4.96, and 5.66% ±0.78 level of cFos colocalization with kisspeptin, POMC and AgRP, respectively (Fig. 24). There were no significant differences between rostral, middle and caudal regions for any of the populations. Off-feed control females showed increased cFos co-localization in both AgRP (75% ±10.03) and ARC kisspeptin (26% ±5.27) neurons ($p=0.021$, $p<0.001$), but not POMC neurons. In contrast, leptin treatment or feeding state did not induce cFos colocalization with POA kisspeptin neurons and GnRH neurons.

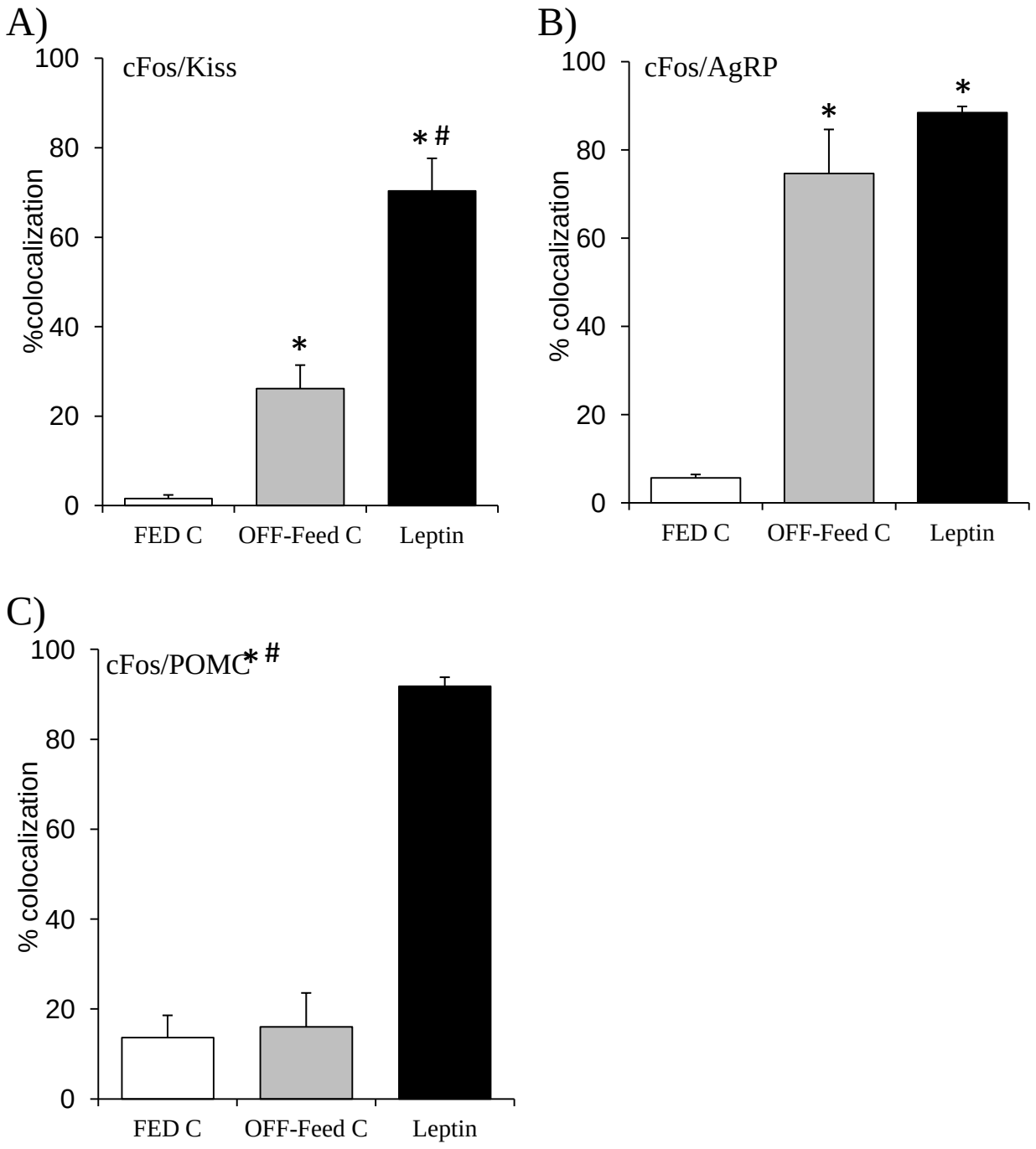

Figure 23. Mean (±SEM) percentage of A) kisspeptin B) AgRP and C) POMC cells colocalizing cFos in the arcuate nucleus in control fed (FED C), control off-feed (OFF-Feed C) and leptin treated females. * indicates significant difference from fed controls, # indicated significant difference from off-feed controls.

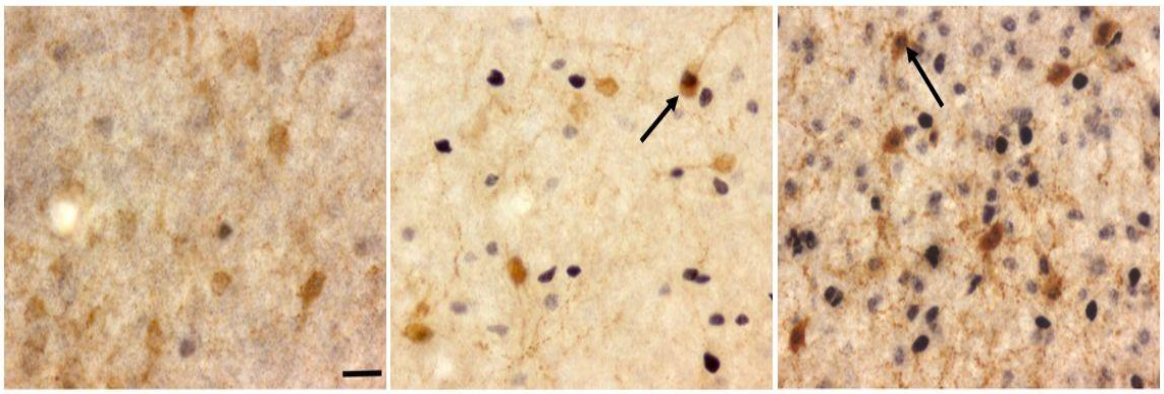

Figure 24. Representative image of ARC KNDy neurons colocalized with cFOS in (A) Control FED, (B) Control OFF FEED and (C) Leptin treated females. Arrows indicate double labelled neurons. Scale bar is 20µm.

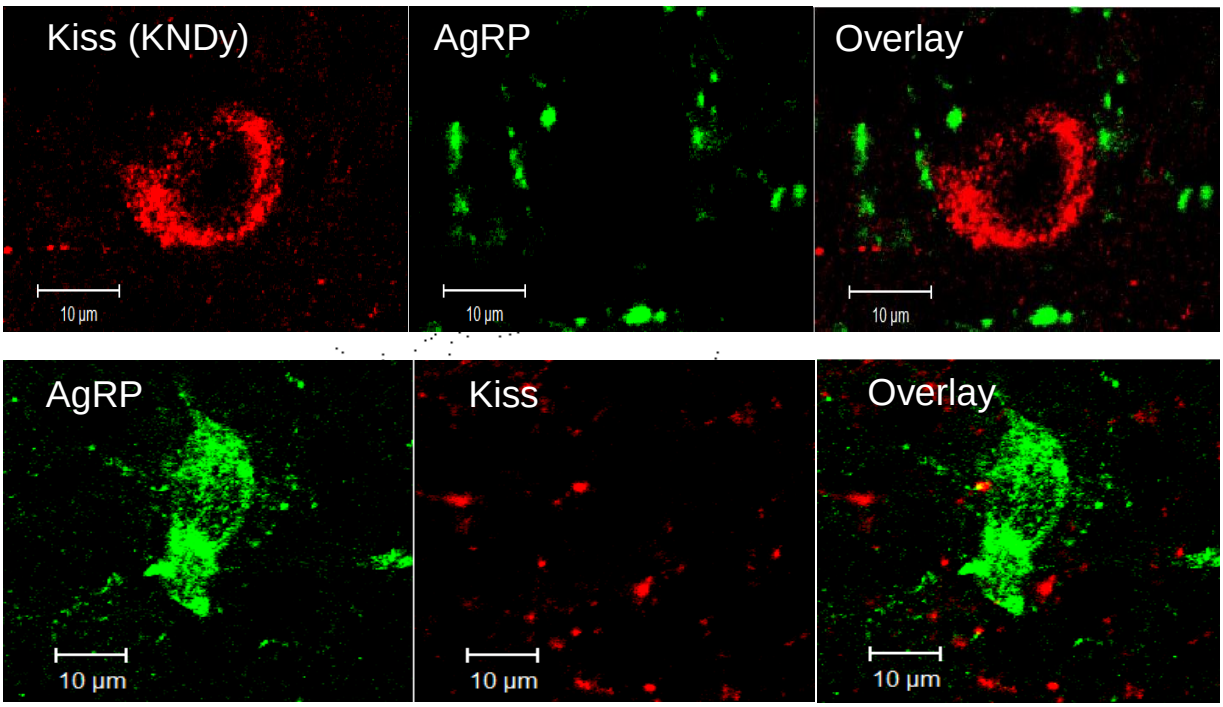

Figure 25. Confocal image showing close contacts between arcuate kisspeptin/KNDy (red) and AgRP (green) cells. Panel (1) shows AgRP close contacts on kisspeptin (KNDy) cell; and panel (2) shows Kisspeptin (KNDy) close contacts onto AgRP cell. Scale bar=10µm.

5.5 Discussion

Findings from this study show that in the ewe, AgRP and POMC neurons are directly activated by leptin (as indicated by induction of pSTAT3 in these populations), while ARC kisspeptin neurons are indirectly activated by leptin (as indicated by induction of cFos in these neurons in the same animals). Moreover, we show that ARC kisspeptin and AgRP neurons are activated during states of negative energy balance.

5.5.1 Leptin-induced activation of ARC kisspeptin, AgRP and POMC neurons

Given the significant influence of leptin on the reproductive neuroendocrine system, it was of interest for us to examine kisspeptin neurons as potential targets of leptin signalling. It was recently shown that in the sheep, no GnRH neurons and <8% of ARC kisspeptin neurons are activated by leptin (as indicated by pSTAT3)(*38*). Indeed, it has been shown in other species that GnRH neurons do not colocalize the leptin receptor (*17*). Therefore, these results are consistent with the view that leptin's influence on GnRH neuroendocrine secretion and the reproductive axis must be indirect via inputs from leptin-sensitive afferent neurons. The effects of leptin on kisspeptin neurons in the sheep differ from the data in the mouse, where 40% of kisspeptin neurons co-express LepRb (*45*), but consistent with transgenic mouse experiments that have shown only 5-6% colocalization of kisspeptin with LepRb (*46*). In the present study, we show that leptin treatment induces a 40% increase in cFos colocalization with ARC kisspeptin neurons when compared to fed control females. This suggests a role for the ARC

kisspeptin neurons in metabolic homeostasis and is consistent with our hypothesis that kisspeptin neurons are activated by leptin, but this activation is indirect and mediated by upstream neurons.

The ARC AgRP and POMC neurons (orexigenic and anorexigenic, respectively) have been proposed as one of the main targets of leptin's actions (*47-48*). Indeed, leptin activates POMC (*35*) and inhibits AgRP neurons (*49*). In this study, we show that leptin induces pSTAT3 colocalization in ~30% of POMC neurons and ~84% of AgRP neurons, suggesting a direct action of leptin on these neurons. This activation was confirmed by cFos colocalization within 88% of AgRP and 92% of POMC neurons in the same animals. The difference in colocalization between pSTAT3 and cFos within the respective neuron populations may stem from various sources. One explanation may be that leptin treatment activates numerous neuronal populations throughout the ARC and other regions that may in turn activate a majority of both AgRP and POMC neurons. Leptin receptors are widespread throughout hypothalamic nuclei including the paraventricular, periventricular, and supraoptic nuclei (*50*). In the sheep, leptin induces pSTAT3 in the ventromedial and dorsomedial hypothalamus, as well as in the ARC (*38*). A recent study has shown that leptin can act in the ventral tegmental area (VTA) to induce pSTAT3 expression in the MBH, suggesting an integrated leptin signaling pathway between these two regions (*51*). Moreover, the ventral premammillary nucleus has been linked to fasting-induced changes in leptin levels that regulate luteinizing hormone secretion (*52-53*). Therefore, leptin targets neurons in multiple areas in the brain, and it is likely that these multiple populations act in concert with each other;

however the specific role(s) played by each in the pathways by which leptin influences the reproductive axis merits further investigation.

5.5.2 Effects of metabolic state on AgRP and POMC activation ?

In this study we show that a 24 hours food restriction increases cFos expression within AgRP, but not POMC neurons, suggesting an activation of the orexigenic pathway induced by a state of low energy balance. This is consistent with previous findings showing that nutritional status can modulate AgRP and POMC activity; AgRP activity is increased after a period of fasting (*54-56*), while POMC activity is increased during satiety or refeeding after a period of fasting (*57*). This nutritional information can be relayed to the kisspeptin neurons and the reproductive axis via direct connections from both AgRP (Fig. 26) and POMC neurons (unpublished data) and vice versa (*58-59*). Another possibility is that AgRP and POMC directly modulate GnRH secretion. Direct contacts from both POMC (*60*) and NPY (colocalized with AgRP) (*61-63*) neurons to GnRH have been previously described in rodents and sheep as well as the effects of these neuropeptides on GnRH neuron excitability (*64-65*). Although this is a possibility, the increased cFos colocalization we observed within ARC kisspeptin neurons in leptin-injected ewes suggests that projections from this subpopulation to GnRH neurons (66) play a role in relaying metabolic information as well.

5.5.3 Effects of metabolic state on kisspeptin activation

A 24 hour off-feed regimen resulted in a >25% induction of cFos colocalization with ARC kisspeptin neurons when compared with fed control females. This suggests that

ARC kisspeptin neurons are activated during states of negative energy balance and may participate in the control of metabolic homeostasis. Studies in rodents (*66-67*) and primates (*68*) have shown that food deprivation reduces Kiss-1 mRNA immunoreactivity. However, we observed no differences in the numbers of kisspeptin immunoreactive neurons between fed and off-feed controls. Although changes in peptide expression do not always parallel changes in activation, our results showing an increase in cFos colocalization with ARC kisspeptin neurons were different than what has been previously noted in the literature. Future experiments will determine whether the other two neuropeptides co-expressed with kisspeptin in the ovine ARC (NKB and dynorphin) are altered by short-term food deprivation. In mice, short-term fasting (48 hrs.) decreases Kiss-1 and Tac2 mRNA during puberty (*69*), suggesting that regulation of NKB is closely linked to that of kisspeptin. The orexigenic neuropeptide dynorphin (*70*), however, appears to be regulated differently. Pre-prodynorphin mRNA is significantly increased after a 24 hour fast in areas of the hypothalamus involved in energy homeostasis (*71-72*). Moreover, there is evidence that dynorphin may mediate the orexigenic effects of NPY in rodents (*73*). In addition, selective blockade of kappa-opioid receptors (the primary receptor for dynorphin) significantly reduces fasting-induced hyperphagia in rats (*74*) and reduces food intake and body weight in obese rodent models (*75-76*). As such, an increase in cFos with kisspeptin/KNDy neurons may reflect an increase in dynorphin activation. It is also conceivable that there is increased influence of melanocortins during the anestrous season and this is causing an increase in kisspeptin/KNDy neuronal activation. In fact, POMC mRNA is increased in anestrous

ewes (*77*) and melanocortins stimulate the reproductive axis (*78-79*), providing a mechanism where an increase in POMC activation can induce an increase in kisspeptin activation.

It is probable that differences in digestive systems (polygastric/ruminant vs. monogastric) could account for some inconsistencies among similar studies examining the activity of ARC kisspeptin neurons following food restriction (increased vs. decreased expression). Indeed, monogastrics (rats and primates) are more acutely responsive to short-term changes in dietary energy intake than ruminants. For example, both chronic under-nutrition and acute fasting result in a rapid suppression of the GnRH system and a cessation of LH pulsatility in monogastrics (*80*). Nonetheless, LH pulsatility can be inhibited when prepubertal heifers (*81*) and rams (*82*) are made hypoglycemic under short term fasting conditions. Moreover, mature cows show metabolic responses to acute feed deprivation such decreased leptin mRNA transcription in adipose tissue and decreased plasma concentrations of leptin and insulin, but no reduction in pulsatile secretion of LH (*83*).

5.5.4 Conclusion

In the present study we show that leptin treatment directly activates the AgRP and POMC neurons and indirectly activates the kisspeptin neurons in the arcuate nucleus of the sheep. We also show that short term food deprivation activates the ARC kisspeptin (KNDy) neurons, possibly implicating this population in metabolic control of reproductive function. As such, we propose that leptin's influence on the reproductive

axis and GnRH secretion involves a pathway comprised of arcuate AgRP and POMC neurons as well as KNDy neurons. Also important to note is that the activation of KNDy neurons appears to be biphasic in response to energy balance. That is, both negative energy balance (short term food restriction) and positive energy balance (leptin) induce activation (cFos) of KNDy neurons. Considering the crucial role that these neurons play within the reproductive axis, it is tempting to speculate that this type of biphasic activity pattern plays an important role in the inhibition of reproductive function during times when energy levels are not ideal. Nevertheless, it is evident that the reproductive and metabolic axes are tightly linked and may involve many intermediary neuronal populations, one of which appears to be the KNDy neurons. Furthermore the leptin signalling pathway within the arcuate nucleus is complex and may involve the contribution of additional metabolic signals. Understanding the neural circuitry involved in this pathway and the neuronal populations that could be involved in this process remains to be answered in future studies.

Appendices:

Appendix 2. *Antibody Controls*

The specificity of the antibody used for pSTAT3 was verified by preabsorption of the diluted primary antibody (1:100) with immunizing peptide (Cell Signaling) at a concentration 5g/ml (Fig. 26), which effectively eliminated all nuclear immunostaining.

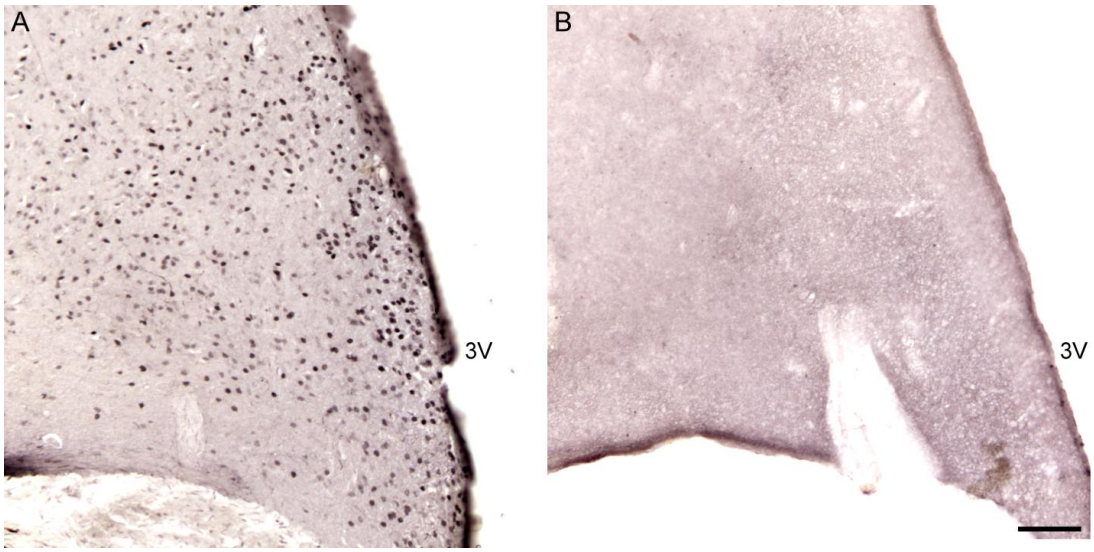

Figure 26. pSTAT3 immunoreactivity in the ovine ARC of a leptin-injected ewe (A) and following preabsorption of the diluted antibody with the peptide antigen at a concentration of 5µg/ml (B). Scale bar = 100µm.

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Chapter 6: Discussion

For this dissertation, I successfully completed a collection of studies to examine the effects of prenatal exposure to testosterone (T) on hypothalamic neurons involved in the regulation of the reproductive and metabolic functions in the sheep.

6.1 Overview

Throughout these studies, I examined the effects of prenatal T treatment on androgen receptor (AR) and insulin receptor (IR) expression with the reproductive and metabolic neurons of the hypothalamus, the ARC KNDy, AgRP and POMC neurons, the POA kisspeptin neurons, and the GnRH neurons. I showed that prenatal T treatment increases AR-immunoreactivity (ir) in the ARC and POA as well as colocalization of AR with KNDy and AgRP and POA kisspeptin neurons. In addition, prenatal T treatment decreases IR colocalization with KNDy and AgRP neurons, but not POMC neurons, while POA kisspeptin and GnRH neurons do not colocalize IR. I also showed that prenatal, but not postnatal, interventions blocking androgen action or increasing insulin sensitivity can prevent the prenatal T induced alterations in KNDy and AgRP neurons. Lastly, I showed that leptin indirectly activates KNDy neurons through a pathway that may include the leptin-sensitive AgRP and POMC neurons. Overall, these results indicate that prenatal exposure to T increases androgen sensitivity and decreases insulin sensitivity in the reproductive and metabolic neurons of the ARC and highlights the importance of prenatal interventions in preventing these effects. These changes may

contribute the reproductive and metabolic dysfunctions associated with the PCOS phenotype.

6.2 Effects of Prenatal T treatment on AR-ir in the brain

In the sheep, measuring testosterone levels has proved to be challenging due to the lack of sensitive assays available (*Padmanabhan, observation*). Moreover, It has been proposed that total serum testosterone measurements are unreliable in screening to detect the presence of hyperandrogenemia (*1*). As such, examining AR-immunoreactivity in the brain provides us with a measure of functional hyperandrogenemia in the adult sheep. The findings of this study reveal that adult females, prenatally exposed to T show an increased number of AR-ir neurons in the POA and ARC as well as increased AR-ir in ARC and POA kisspeptin and ARC AgRP neurons. This increase in AR-ir appears to be programmed by the androgenic action of T, as prenatal treatment with DHT mimics the effects of T while prenatal co-treatment with androgen antagonist (TF) bocks these effects. These sex differences, along with the effects of prenatal T treatment suggest a prenatal masculinization of the female brain by testosterone, which then influences reproductive and metabolic neuroendocrine function and behavior in adulthood.

6.2.1 Does increased AR-immunoreactivity reflect organizational or activational effects of testosterone?

Treatment of the pregnant mother with testosterone (T) from days 30–90 of gestation increases fetal exposure to androgens as well estrogens (*2*). As such, it is reasonable to propose an organizational role for testosterone, possibly laying the framework for dysfunction in reproductive and metabolic function during adulthood. Although

measuring T levels in adult female sheep is problematic, it is reasonable to postulate that early life alterations in neuroendocrine function and masculinization of various systems may be maintained by high levels of circulating T.

Although control rams displayed the highest AR-ir in all brain areas analyzed, castrated rams had comparable or lower numbers of AR-ir neurons than control ewes. It is difficult to say whether the increase in AR-ir that we observed in the adult sheep brain is an indication of higher circulating androgens or an organizational effect of exposure to T. Prenatal T treated ewes and controls were ovariectomized, removing the main source of androgens (*3*). However, when compared with castrated males, who were exposed to high T levels during gestations, prenatal T treated ewes displayed higher AR-ir in the brain. This suggests a few possibilities: 1) an activational effect of androgens in prenatal T treated females stemming from an adrenal source (*4*) (which has been shown to be higher in females than males, albeit during fetal life) (*5*) or from local biosynthesis of neurosteroids in the brain (*6*), or 2) an organizational effect of testosterone that is more pronounced in the female, rendering them more sensitive to circulating androgens, or 3) a combination of these factors. Regardless, we cannot dismiss a role for the adrenal glands in the contribution of androgens, an influence that is significantly increased in prenatal T treated sheep (*4*) and primates (*7-8*) as well as women with PCOS (*9-11*). However, adrenalectomy is not feasible in our current sheep model, leaving this question to be addressed in other animal models. Future studies could also address the activational effects of testosterone by exogenous administration of androgens during adulthood and examining AR-ir in the brain. Comparing females treated with T during adulthood with

ovariectomized prenatal T treated females might be useful to help determine whether organizational/permanent or activational/transient are responsible for increased AR-immunoreactivity in the brain.

6.2.2 How does functional hyperandrogenemia contribute to the PCOS phenotype in prenatal T treated sheep?

Hyperandrogenism is one of the most common PCOS traits. We show that prenatal T treated sheep display functional hyperandrogenemia, manifested as an increase in AR-ir during adulthood, suggesting a masculinization of the brain. Functional hyperandrogenemia has also been shown at the level of the ovaries and adrenals in prenatal T treated sheep (*12-13*) and monkeys (*8, 14*). This masculinization, although not as pronounced as in the ram, likely influences reproductive, metabolic functions and social behaviours. Indeed, prenatal T treated ewes display masculinized modes of tonic and surge secretion of LH (*15-16*) and masculinized social behaviours (*17*). In support of this, androgens have been shown to inhibit the negative feedback effects of estrogen and progesterone on pulsatile LH secretions (*18*).

6.2.3 Linking hyperandrogenemia and hyperinsulinemia

Adult prenatal T treated sheep are hyperinsulinemic due to metabolic disruptions which result in decreased insulin sensitivity (*19-22*). Hyperinsulinemia exacerbates hyperandrogenemia by acting together with elevated LH to increase androgen production in theca cells of the ovary (*23-26*). Insulin can also suppress sex hormone binding globulin (SHBG) produced by the liver, resulting in an increase in unbound plasma testosterone (*27*). Moreover, weight gain can contribute to the increase in circulating

androgens. Human adipose tissue can synthesize androgens by means of 17β-hydroxy steroid dehydrogenase (17βHSD), an enzyme found in the liver, skin, and adipose tissue that catalyzes the conversion of Androstenedione (A) to T (*28*). This enzyme has been shown to have increased expression in obese women (*29*).

The insulin-androgen feedback loop has also been shown to work the opposite way, where increased androgens promote hyperinsulinemia. For example, pregnant ewes treated bi-weekly with T show an increase in circulating insulin levels, which is believed to be a direct effect of testosterone (*Abi Salloum, B. and V. Padmanabhan, unpublished data*). Thus, the positive feedback loop between hyperinsulinemia and hyperandrogenemia may contribute to the propagation of the PCOS phenotype and increases its severity over time. This is reinforced by the studies showing improvements in hyperandrogenemia and ovulatory function with treatments that decrease insulin levels or improve insulin signalling (*30-33*). Nevertheless, the relationship between hyperinsulinemia and hyperandrogenism is complex and requires further investigation in the context of metabolic and reproductive function.

6.2.4 Evidence for androgen-dependent mechanisms regulating kisspeptin and AgRP activity?

The ARC kisspeptin subpopulation (also known as the KNDy neurons) play an important role in regulating pulsatile and surge secretion of GnRH, while POA kisspeptin neurons are activated only during the GnRH surge (*34-37*). Prenatal T treated ewes display increased GnRH pulse frequency and a dampened and delayed GnRH surge, effects

which may be due to alterations within KNDy neurons. As such, an increase in AR-ir in these neurons, rendering them more sensitive to androgens, may impact GnRH secretion and subsequently, reproductive function. In the male mouse, testosterone acts through both AR and estrogen receptor alpha (ERα) to inhibit kisspeptin (*Kiss1)* (*38*) and Dynorphin *(Pdyn)* expression (*39*) in KNDy neurons. However, in the rostral kisspeptin population, which includes the anteroventral periventricular nucleus (AVPV), T stimulates the expression of *Kiss1* mRNA (*39*). These findings suggest that kisspeptin neurons are direct targets for T, which likely plays a role in mediating both pulsatile and surge mechanisms of GnRH secretion. Furthermore, Navarro *et al.* (2011) showed that sham-treated AR knock-out (KO) mice had higher levels of *Kiss1*, *Tac2 (*NKB*)*, and *Pdyn (*Dynorphin*)* mRNA in the ARC compared to wild-type (WT) controls, suggesting that androgens are important during the developmental period of KNDy neurons (*40*), possibly regulating peptide expression during this sensitive period (*41*). Previous work from our lab has shown that prenatal T treated ewes show a peptide imbalance in KNDY neurons, with a reduction in dynorphin and NKB expression, but not kisspeptin (*42*). Unpublished data from our lab indicates that prenatal co-treatment with Flutamide (F, androgen blocker) largely reverses the effects of prenatal T on NKB and dynorphin, suggesting that this peptide imbalance is largely organized by the androgenic actions of T (*Lehman, unpublished*) and these alterations are sustained into adulthood.

The increase in AR colocalization within AgRP neurons suggests a means through which testosterone may act to modulate peptide expression in this ARC population as well. As

previously discussed, prenatal T treated ewes show an increase in the number of immunoreactive AgRP neurons compared to control ewes (*43*) and decreased colocalization with insulin receptor (IR) (*44*). Prenatal co-treatment with Flutamide reverses these effects on AgRP peptide and IR colocalization, suggesting that these changes are organized by the androgenic actions of T (acting on AR) (*43*). The effects of T on this population have also been shown in rodents and sheep. In male sheep, NPY (an orexigenic neuropeptide which is largely co-localized with AgRP (*43*, *45-46*)), expression is increased during a long-day (LD) photoperiod in a T-dependent manner (*47*). In castrated male rats, T replacement increases NPY mRNA, suggesting regulation of this peptide by testosterone (*48*). Regulation of NPY/AgRP has also been found in humans, where anti-androgen treatment in patients with prostate cancer decreases NPY levels (*49*). One possibility is that prenatal exposure to T increases AR colocalization with AgRP, and this change lays the framework for testosterone actions in adulthood altering AgRP immunoreactivity and IR colocalization in this population. This, in turn, may contribute to the insulin resistance and increased risk of obesity observed in the prenatal T sheep model (*21-22*).

6.2.5 Significance and future studies

Since androgen excess is one of the main features of PCOS, it was of great importance to determine whether our prenatal T treated sheep displayed hyperandrogenemia. Results presented in Chapter 2 are the first to show that, in the sheep, prenatal T treatment results in functional hyperandrogenism at the level of the brain. Although we showed that

prenatal T treatment increases AR-ir in areas of the ewe brain that are critical for control of reproductive and metabolic functions as well as sexual behavior, the mechanism by which prenatal T alters postnatal development of these systems is not yet known. One possibility is that prenatal T masculinizes the female brain, rendering it more sensitive to circulating androgens and thus, altering control of neuroendocrine function in the adult female. Prenatal T exposure could also lead to abnormal programming of androgen producing tissues, such as the ovaries and adrenals, resulting in increased circulating androgens. Therefore, the increased AR-ir seen in the prenatal T females could be a programmed prenatal effect or it could be a reflection of high levels of circulating androgens, or a combination of both factors. Because our animals are ovariectomized and estradiol replaced, we can exclude androgen contribution from the ovaries; however excess androgen production by the adrenals may be a contributing factor to high circulating levels of T. Future studies will aim to determine whether prenatal or postnatal androgens induce an increase in AR-ir in the brain. It would also be of interest to examine AR-ir in the sheep brain at major developmental stages, including during the prenatal and prepubertal period, with the goal to identify additional stages of brain organization and timepoints for interventions with anti-androgen treatments.

6.3 Effects of prenatal T treatment on IR colocalization with KNDy and AgRP neurons

Insulin serves as a signal between the metabolic and reproductive systems, communicating energy availability to the hypothalamus and thereby affecting

reproductive mechanisms. Prenatal T treated sheep display insulin resistance and hyperinsulinemia, factors which may contribute to the reproductive and metabolic dysfunctions seen in these females. In experiments of Chapter 3, we found that 1) ARC KNDy, AgRP and POMC, but not POA kisspeptin or GnRH neurons colocalize insulin receptor (IR), 2) prenatal T treatment reduces IR colocalization with KNDy and AgRP neurons and 3) this effect is blocked by prenatal T co-treatment with anti-androgen, but only in the AgRP population. Hence, different mechanisms are involved in the organization of IR in the two different neuronal populations, AgRP and KNDy neurons, via androgenic and estrogenic actions of T, respectively.

6.3.1 What is the functional impact of the decrease in IR colocalization within KNDy and AgRP neurons?

Prenatal T treated ewes show evident insulin resistance and hyperinsulinemia (*21-22*), as well as other reproductive and metabolic dysfunctions, similar to those seen in women with polycystic ovarian syndrome (PCOS). Together, infertility and insulin resistance are a major concern in women with PCOS and are exacerbated by excess weight (*30, 50-52*). The decrease in IR colocalization with the KNDy and AgRP neurons renders these populations less responsive to insulin signaling and in turn, may affect multiple systems which rely on this communication. For example, insulin signaling is important in the initiation of puberty and maintenance of normal reproductive function. As such, women with type I diabetes have an increased risk for reproductive dysfunctions and delayed puberty (*53*). Clinical research suggests that these reproductive disruptions may stem

from abnormalities in the GnRH pulse generator and not at the level of the pituitary (*53*). Recent findings from studies of transgenic mice reveal that cell-specific deletion of insulin receptors from kisspeptin neurons caused a delay in onset of puberty, but did not affect adult fertility (*54*). Moreover, male and female mice with GnRH specific IR deletion display normal pubertal timing and fertility (*55*). These studies suggest that IR expression with kisspeptin or GnRH neurons is not essential for the maintenance of reproductive function in mice; however IR expression with kisspeptin plays a critical role in the initiation of puberty. Nevertheless, a role for insulin signalling in the regulation of GnRH secretion cannot be dismissed. For example, diabetic sheep with low peripheral insulin, display increased pulsatile LH secretion following central insulin supplementation (*56*). As such, insulin regulation of GnRH secretion may involve other insulin-responsive sets of neurons, such as AgRP (*57-58*) and POMC (*59-60*), which have been shown to influence GnRH activity. Additional studies are required to investigate the role of these populations in conveying insulin signals to the GnRH neuron.

As a future direction, it would be of interest to examine the role of IR expression with KNDy as it pertains to the onset of puberty in the sheep. The role of NKB and kisspeptin in the initiation of female puberty has received considerable attention (*61-63*) and may involve the conversion of multiple signals, including insulin. The group of females analyzed in our study (ovary intact up to 2 years of age, then OVX) do not present alterations in the onset of puberty (*64*); however, prenatal T treated sheep that have been OVX and E-replaced shortly after birth display advanced puberty onset (*65*). Future studies might compare IR/KNDy colocalization between the OVX+E replaced and ovary

intact sheep models and in prenatal T females co-treated with insulin sensitizer. The OVX+E replaced prenatal T treated sheep may provide us with a model for studying pubertal onset in PCOS, as premature puberty is the earliest manifestation of this disorder (*66-67*).

The significant but relatively modest decrease in insulin receptor colocalization within AgRP neurons suggests that other aspects of the insulin signaling pathway may mediate the dramatic increase in AgRP peptide expression seen in this animal model. In addition to alterations in IR at the level of the brain, prenatal T treated sheep also show decreased IR in the liver (*68*). Considering the modest decrease in IR colocalization with AgRP in these animals, it is unlikely that the reduced IR in the liver is a direct result of central IR decrease. Nonetheless, this provides us with a peripheral component of insulin signalling that is also altered by prenatal T treatment and together, these prenatal T induced changes may contribute to the insulin resistance in this animal model. In transgenic mice where IR are specifically knocked out of AgRP neurons, hepatic glucose production is increased in response to insulin (*69*). When IR is restored in these neurons, hepatic glucose production is decreased in response to insulin (*70*), suggesting that coexpression of IR with AgRP neurons in mice is an important modulator of hepatic glucose production. It is also possible that downstream signaling molecules in the insulin receptor pathway are altered by prenatal T treatment. In addition to decreased IR expression in the liver, prenatal T treated sheep show a decrease in IRS-II, AKt, mTOR, PPAR-γ mRNAs in this organ, all important components of the insulin pathway (*68*). Investigating the downstream insulin

signaling pathway in AgRP/NPY neurons would be beneficial to further understanding the insulin resistance in the brain conferred by excess prenatal testosterone exposure.

6.3.2 What is the significance of the absence of IR from POA kisspeptin and GnRH neurons?

In the sheep, colocalization of IR with GnRH has not been previously examined. Our results show that GnRH neurons in the POA, AHA or MBH do not colocalize IRβ. This results is in contrast to data in the mouse where insulin may regulate reproductive function by directly stimulating GnRH gene expression (*71*). Thus, insulin signaling to GnRH neurons in the sheep likely involves inputs from afferent insulin sensitive neurons. Similarly, we showed that IR is not colocalized with POA kisspeptin neurons, further distinguishing this population of kisspeptin neurons from the ARC KNDy population. There are several other marked differences between the POA and ARC kisspeptin populations, which are consistent with their functions. Unlike the POA neurons, the ARC kisspeptin neurons (KNDy) are glutamatergic and co-express two other neuropeptides-dynorphin and NKB (*36*), each playing a different role in the regulation of GnRH pulses (*34*). Within the ARC, KNDy neurons also lie in close anatomical proximity to AgRP and POMC neurons, and direct connections between these three populations have been described (*72-74*). Differences between ARC and POA kisspeptin neurons have also been noted in their activation by insulin. In the sheep, a bolus injection of insulin (4 IU/Kg) during late follicular phase induces cFos activation in ARC, but not POA, kisspeptin neurons (*Fergani, C. et al. in press*). As such, the KNDy neurons are well suited to act as

a mediator by which insulin can influence the reproductive neuroendocrine function and regulate GnRH secretion.

6.3.3 Significance and future studies

This group of experiments reveals that at the level of the brain, insulin receptor colocalization within reproductive (KNDy) and metabolic (AgRP) neurons is decreased as a result of prenatal T treatment. A reduction in insulin sensitivity in these neurons may contribute to insulin resistance and hyperinsulinemia in the prenatal T sheep model and may subsequently influence reproductive function during adulthood. In addition to changes at the level of the brain, it has previously been shown that prenatal T treatment results in decreased IR in the liver and muscle (*68*), which also contributes to peripheral insulin resistance. Considering the strong link between insulin resistance, obesity and reproductive dysfunction (*75-76*), it is reasonable to speculate that a combination of decreased insulin sensitivity at the level of the brain and periphery contributes to the metabolic and reproductive dysfunctions seen in prenatal T sheep and, by inference, in women with PCOS. Because sheep in the present study are on a maintenance diet, normal body weight is sustained with no differences between control and prenatal T females. It would be of interest to examine IR expression with AgRP and KNDy neurons in the diet-induced obesity prenatal T sheep model to determine if changes in IR expression with these neuropeptides are associated with exacerbation of the metabolic and reproductive phenotype linked to obesity (*21*).

6.4 Effects of prenatal and postnatal interventions

This set of experiments suggests a neuroendocrine basis for the beneficial effects of therapies that are already used in the treatment of women with PCOS. The anti-androgen Flutamide (F) and/or the insulin sensitizer, Rosiglitazone (R) are often used to alleviate symptoms associated with PCOS. We show that prenatal, but not postnatal, interventions with these therapies were able to block the effects of prenatal T on AR and IR colocalization with KNDy and AgRP neurons, and AgRP neuronal numbers.

6.4.1 Effectiveness of prenatal and postnatal interventions with Flutamide or Rosiglitazone in blocking the effects of prenatal testosterone

The ability of pre- but not post-natal interventions with insulin sensitizer to block the effects of prenatal T treatment on the IR and AR colocalization with KNDy and AgRP neurons suggests that the prenatal period may provide a critical time point to intervene and block the effects of prenatal T seen later during adulthood. Nonetheless, the postnatal period may also provide us with an additional period for effective intervention. For example, postnatal treatment with the insulin sensitizer Rosiglitazone (R) has been shown to improve reproductive function in prenatal T treated sheep (*77*), reducing the deterioration in number of cycles from 80% to 20% in the second breeding season. In another study, postnatal treatment with F and R was able to reverse the effects of prenatal T on some aspects of the preovulatory LH surge (*78*). Prenatal T treated ewes that received postnatal F or R treatment (T+F or T+R) showed an increase in LH surge magnitude, which is typically dampened by prenatal T treatment (*78*). The timing of the surge, however was still delayed, suggesting that the prenatal and postnatal period of

development are important for the programming of different aspects of the LH surge mechanism (*78*).

In women with PCOS, insulin sensitizers are commonly used to alleviate some of the symptoms associated with hyperinsulinemia and hyperandrogenemia (*79-82*). As such, it is surprising that postnatal treatment with R did not reverse the effects of prenatal T-treatment on the KNDy and AgRP populations in our animals. It may be that the organization of IR and AR expression in these populations is important during the early stages of development in order to set the framework for reproductive and metabolic functions later in life. In this regard, it is interesting that although interventions with androgen antagonist (*83*), insulin sensitizer (*84-86*), or combined (*87-88*) are able to improve ovulation rate in subjects with PCOS, optimal success with pregnancy rate are yet to be realized. It is possible that postnatal interventions are able to alleviate some dysfunctions associated with PCOS; however some neuroendocrine mechanisms remain permanently altered by prenatal T treatment and set the critical parameters for successful reproductive and metabolic function to a higher level.

6.4.2 Future studies

The ability of prenatal treatment with insulin sensitizer to block the effects of prenatal T on IR colocalization within KNDy and AgRP neurons suggests that insulin signalling, whether occurring via a mechanism involving androgen signalling or alone, can program some parameters of the neuroendocrine axis in prenatal T treated sheep. For example, insulin has been shown to inhibit aromatase (an enzyme which converts maternal or fetal adrenal androgens to estrogens) activity in human cytotrophoblasts (*89-90*). Therefore,

hyperinsulinemia in pregnant ewes treated with T or women with PCOS may increase the androgenic actions of testosterone to which the female fetus is exposed (*91*). Moreover, The ability of prenatal treatments with either insulin-sensitizer or anti-androgen to block the effect of prenatal T indicate that a common mediator involving both androgen and insulin signalling is responsible for the long-term alteration in the AgRP population. One possibility may be that a common downstream component in the androgen and insulin signalling cascades is altered by prenatal T exposure. One example is Foxo1, a transcription factor that plays a key role in insulin-mediated regulation of metabolism (*92*). Foxo1 upregulates IR and activates insulin signalling (*93*), while having an inhibitory effect on androgen induced AR signalling (*94*). Moreover, by blocking Foxo1 inhibition of AR signalling, insulin can exert a stimulatory effect on AR (*94*). As such, alterations in Foxo1 activity can disrupt both androgen and insulin signalling. Nevertheless, the complex interactions between insulin and androgen signalling require further investigation. Future studies will examine alterations in insulin and androgen signalling components in order to define possible mediators which may simultaneously influence both signalling pathways. Moreover, future studies will combine prenatal and postnatal interventions targeted at blocking androgen action or increasing insulin sensitivity or both, as combined treatments have proven the most effective in improving ovulation rates and insulin sensitivity in women with PCOS (*88*).

6.5 Direct and indirect activation by leptin in the ARC

Our lab has previously shown that in the sheep, leptin does not directly activate kisspeptin (ARC or POA) or GnRH neurons (*95*). However, leptin's influence on GnRH secretion and reproductive function has been widely described (*96-97*). This set of experiments expands on the previous findings by showing that in the sheep, AgRP and POMC neurons are directly activated by leptin (as indicated by induction of pSTAT3 in these populations), while ARC kisspeptin neurons are indirectly activated by leptin (as indicated by induction of cFos in these neurons). Moreover, ARC kisspeptin and AgRP neurons are activated during states of negative energy balance.

6.5.1 Leptin signalling pathways in the arcuate: communication between the ARC AgRP, POMC and KNDy neurons

The KNDy, AgRP and POMC neurons form a complex network within the arcuate (*72-74, 98*). POMC and NPY/AgRP neurons mediate a diverse range of leptin's actions in metabolism (*99-104*) and several studies have identified these populations also as key players linking leptin action and reproduction. As such, leptin's influence on reproductive function and GnRH secretion may involve a pathway that includes the metabolic AgRP and POMC neurons as well as the KNDy neurons (*95*). It has been previously shown that the melanocortins (POMC derivatives) communicate with kisspeptin to relay the stimulatory influence of leptin to the reproductive axis (*59, 98*). Conversely, NPY has been shown to have an inhibitory influence on reproductive function (*105*). AgRP administration or pharmacologic blockade of the melanocortin 4 receptor (MC4R)

dampens the LH surge in female rats (*106-107*) and suppressed pulsatile release of LH in ovariectomized rhesus monkeys (*108*). Nevertheless, the influence of POMC and AgRP/NPY on reproductive function merits further investigation. For example, humans with loss-of-function mutations of melanocortin signaling show no reproductive deficits (*109-111*). In addition, mice with specific deletion of LepRb from POMC neurons, AgRP neurons, or both, show no reproductive dysfunctions (*112*). Therefore, our findings can be explained in two ways: 1) there exist non- POMC, non-AGRP/NPY neurons that express LepRb in the ARC and contribute to the control of feeding and reproductive function; and/or 2) LepRb expressing neurons outside the ARC significantly contribute to leptin's actions on feeding and reproduction. The identity of non-POMC, non-AGRP/NPY leptin sensitive neurons in the ARC remains to be established; however there are numerous extra-ARC sites that contain leptin responsive neurons (*113-115*). Leptin may influence kisspeptin activity via projections from adjacent nuclei. One example is the ventral premammillary nucleus (PMV), a hypothalamic neuronal group that has been shown to play a critical role in leptin's action on reproductive control (*95, 116-118*). In the rat, the PMV houses a high density of LepRb expressing neurons (*119-120*), most of which have been shown to directly innervate GnRH neurons (*118*). It would be of interest to examine the role of the PMV in mediating leptin's influence on reproductive function in the sheep, and specifically its role with the AgRP, POMC, KNDy signalling pathway.

Although we are proposing a pathway that comprises of direct activation by leptin of AgRP and POMC, and subsequently KNDy and GnRH, we cannot rule out the possibility of direct modulation of GnRH by POMC and NPY neurons. In rodents, POMC and

AgRP/NPY fibers innervate GnRH neurons (*60, 121-123*) and can directly regulate GnRH neuronal excitability (*58*), providing a direct pathway through which metabolic signals can regulate reproductive function.

6.5.2 Increased ARC kisspeptin (KNDy) neuronal activation after a short-term food deprivation

Studies have implicated kisspeptin in mediating nutritional control of reproduction. Food restriction in rodents (*124-125*) and primates (*126*) reduces kisspeptin expression and kisspeptin treatment alleviates undernutrition-induced delayed puberty in female rats (*127*). Thus, our results showing an increase in cFos colocalization within ARC kisspeptin neurons in food restricted ewes was unexpected. However, in the sheep, where the three KNDy neuropeptides (kisspeptin, NKB, dynorphin) are almost 100% colocalized (*36*), an increase in KNDy activation may reflect changes in any of the three neuropeptides. In mice, short-term fasting (48 hrs.) decreases Kiss1 and Tac2 mRNA during puberty (*61*), suggesting that regulation of NKB is closely linked to that of kisspeptin. However, the orexigenic neuropeptide dynorphin (*128*), appears to be regulated differently. Pre-prodynorphin mRNA is significantly increased after a 24 hour fast in areas of the hypothalamus involved in energy homeostasis (*129-130*). Moreover, there is evidence that dynorphin may mediate the orexigenic effects of NPY as selective blockade of kappa-opioid receptors (the primary receptor for dynorphin) reduces fasting-induced hyperphagia in rats (*131-132*) as well as food intake and body weight in obese rodent models (*133-134*). Although the source dynorphin was not established, it is possible that an increase in cFos with KNDy neurons may reflect an increase in

dynorphin activity. Further experiments will address whether short-term fasting differentially alters neuropeptide expression within the ARC KNDy neurons. It would also be of interest to examine the effects of short-term or long-term fasting on KNDy neuropeptide expression in the diet induced obesity sheep model or prenatal T model.

6.5.3 Importance of leptin in women's reproductive health and evidence for dysfunctional leptin signaling in women with PCOS

Obese individuals are hyperleptinemic and leptin resistant (*135-136*). Nonetheless, the exact effects of high leptin levels on reproductive activity in obesity are not well defined. Considering the high prevalence of obesity in women with PCOS (*137*), it is reasonable to assume that leptin signalling is an important component in this disease model and may provide us with a point of intervention to alleviate some symptoms of PCOS. However, studies linking leptin and PCOS have yielded controversial results. One study found that leptin levels are elevated in women with PCOS (*138*), but subsequent studies were not able to reproduce these findings, challenging the concept that changes in leptin levels might play a role in the etiology of PCOS (*139-143*).

Leptin also plays an important role in the initiation of puberty (*144-146*). Premature or early puberty is one of the earliest indications of PCOS (*66-67*). As such, it would be beneficial to examine the role of leptin signalling in the onset of puberty in prenatal T treated ewes. It has been previously shown that the time of neuroendocrine puberty is sexually differentiated in sheep, with males displaying earlier onset of puberty (*65*, *147*).

Moreover, Prenatal T exposure also alters the timing of puberty and this has been shown in female rats (*148*), guinea pigs (*149*), and primates (*150*). In sheep, prenatal T treatment advances the time of the pubertal LH rise in females to an age that is equivalent to the initiation of testicular activity in the male (*65*). However, this observation is only apparent in the ovariectomized (OVX) E-replaced sheep model (where E2 concentrations are sustained at physiological levels using a continuous release device from shortly after birth) and not in the ovary intact prenatal T ewe (*64*). Nevertheless, the OVX-E replaced prenatal T treated sheep may provide us with a model for studying the contributions of leptin signalling in pubertal onset in PCOS.

6.6 Conclusions

For this dissertation I described the effects of prenatal T treatment of the reproductive (KNDy) and metabolic (AgRP) control neurons of the ewe hypothalamus and showed that prenatal exposure to T permanently alters receptor (AR and IR) expression in these populations, rendering them hypersensitive to androgen signalling and hyposensitive sensitive to insulin signalling (Fig. 27). These alterations can be blocked if interventions with insulin sensitizers and androgen blockers are established during the prenatal period. Although postnatal treatments did not reverse the prenatal T induced effects on the KNDy and AgRP neurons, other studies have found promising result with these treatments. As such, it is likely that postnatal interventions are able to alleviate some dysfunctions associated with PCOS; however some neuroendocrine mechanisms remain permanently altered by prenatal T treatment and alter the critical parameters required for

normal reproductive and metabolic function. These findings suggest that disruptions in the prenatal organization of critical hypothalamic parameters result in alterations in the development of the reproductive and metabolic systems of the brain and contribute to the PCOS phenotype during adulthood. Nonetheless, more work is required to understand the complex mechanisms though which prenatal T programs reproductive and metabolic dysfunctions at the level of the brain and periphery.

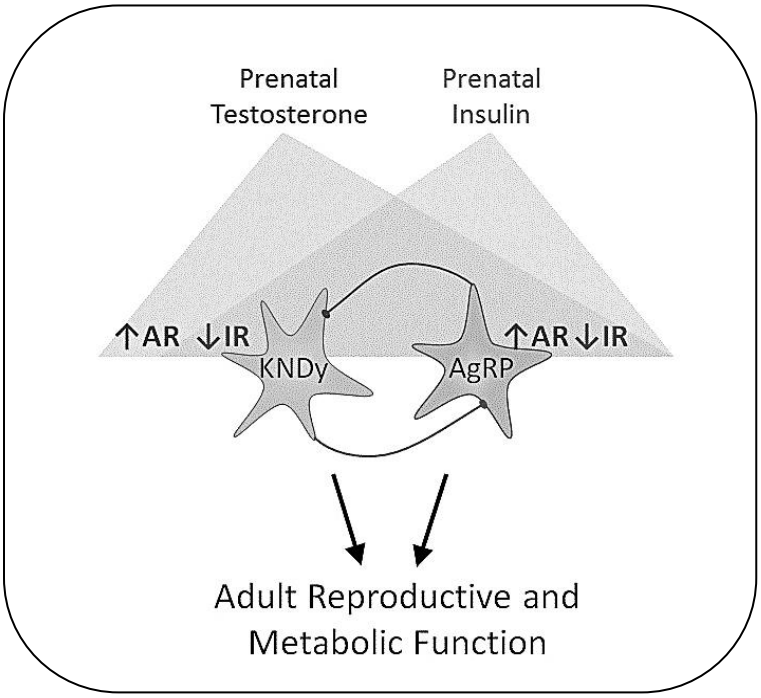

Figure 27. Schematic representation illustrating the organizational effects of excess prenatal Testosterone and Insulin exposure on androgen receptor (AR) and insulin receptor (IR) expression in arcuate (ARC) KNDy and AgRP neurons and the potential impact on adult reproductive and metabolic functions.

PCOS is a complex endocrine disorder in women and a major contributor to ovulatory dysfunction, infertility, obesity, type 2 diabetes mellitus, and cardiovascular disease (*151*). Despite its prevalence, the etiology of PCOS remains unclear. Contributions from prenatal/environmental or genetic factors may result in insulin resistance, abnormal steroidogenesis and hypothalamic–pituitary–ovarian dysregulation. Investigating the dysfunctions associated with PCOS can help us develop appropriate interventions administered at the correct time-point to prevent the onset of PCOS-related co-morbidities. Our hope is that by identifying the basic mechanisms involved in the long term changes induced by prenatal T exposure, we will identify targets for clinical intervention at the level of the brain, as well as periphery. Future studies will aim to prevent and/or reduce the symptoms associated with PCOS as well as transgenerational susceptibility to this disorder by providing appropriate prenatal and postnatal treatments.

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Attended the University of Michigan (department of Molecular and Integrative Physiology) in Ann Arbor, Michigan and the University of Mississippi Medical Center (department of Neurobiology and Anatomical Sciences program) in Jackson, Mississippi as a visiting scholar.

EDUCATIONAL BACKGROUNG

PhD — **June 2011 – August 2013**
University of Western Ontario
Department of Anatomy and Cell Biology
Supervisor: Dr. Michael Lehman

MSc — **Sept. 2009 – June 2011**
University of Western Ontario
Graduate Program in Neuroscience
Supervisor: Dr. Michael Lehman

BSc — **Sept. 2003 – May 2007**
University of Western Ontario
Department of Biology

SCOLARSHIPS

2009-2012- Schulich Graduate Scholarship - University of Western Ontario
Sum of tuition fees up to six terms of a doctorate degree. Awarded to students with an academic average of 80+.

2003-2007- Western Scholarship of Distinction – University of Western Ontario
Admission scholarship: $1000 awarded to students with an academic average of 85+.

AREAS OF EXPERIENCE	DETAILS
Animal Handling	Behaviour watching and recording of sheep, blood sampling from jugular vein, brain tissue removal, perfusion and fixation of brain, organ tissue removal and fixation
Tissue processing	Brain tissue sectioning, immunohistochemistry (enzyme and fluorescence)
Statistical analysis	Quantitative and qualitative analysis of immunostaining, Microscopy: phase contrast, fluorescence, confocal, data statistical analysis and presentation (Microsoft Word, SigmaStat, Excel, PowerPoint, and Image J Software)

PUBLICATIONS

1. Goodman, R.L., Hileman, S.L., Nestor, C.C., Porter, K.L., Connors, J.M., Hardy, S.L., Millar, R.P., **Cernea, M.**, Coolen, L.M., and Lehman, M.N. 2013. Kisspeptin, neurokinin B, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology, In Press.*

2. **Cernea, M**., Coolen, L.M., Padmanabhan, V., Merkley, C., Lee, T., and Lehman, M.N. 2012. Effects of prenatal testosterone on KNDy-GnRH circuitry in a sheep model of PCOS, Endocrinology (*manuscript to be submitted*).

3. **Cernea, M.**, Lee, T., Cheng, G., Padmanabhan, V., Lehman, M.N., and Coolen, L.M. 2012. Excess prenatal testosterone increases androgen receptor immunoreactivity in hypothalamic areas of the adult female sheep brain, Endocrinology (*manuscript to be submitted*).

4. **Cernea, M.**, Phillips, R., Padmanabhan, V., Coolen, L.M. and Lehman, M.N. 2012. Prenatal testosterone decreases colocalization of insulin receptors in KNDy and AgRP neurons in adult ewes: implications for reproductive and metabolic dysfunctions, Endocrinology (*manuscript to be submitted*).

5. **Cernea, M.**, Coolen, L, Phillips, R., and Lehman, M.N. 2012. Neural pathways by which leptin may modulate GnRH secretion in the sheep: activation of AgRP, POMC and kisspeptin (KNDy) neurons (*in preparation*).

6. **Cernea, M.**, Coolen, L.M., Padmanabhan, V., and Lehman, M.N. Prenatal and postnatal contribution of insulin in the programming of androgen and insulin receptor colocalization with KNDy and AgRP cells in the hypothalamus of prenatal testosterone-treated sheep (*in preparation*).

ABSTRACTS

1. **Cernea, M.**, Coolen, L.M., Padmanabhan, V., and Lehman, M.N. Co-treatment with insulin sensitizer or androgen antagonist blocks the effects of prenatal testosterone on AgRP cell number in female sheep hypothalamus. 43rd Annual Society for Neuroscience Meeting in San Diego, CA. Abstract and poster presentation

2. **Cernea, M.**, Phillips, R., Padmanabhan, V., Coolen, L.M. and Lehman, M.N. 2012. Prenatal testosterone decreases co-expression of insulin receptors in KNDy neurons in adult ewes. 2nd Kisspeptin World Conference: Kisspeptin signaling in the brain in Tokyo, Japan. Abstract and poster presentation.

3. **Cernea, M.**, Phillips, R., Padmanabhan, V., Coolen, L.M. and Lehman, M.N. 2012. Prenatal testosterone decreases co-expression of insulin receptors in KNDy neurons in adult ewes. 42nd Annual Society for Neuroscience Meeting in New Orleans, LA. Abstract and poster presentation.

4. **Cernea, M**., Coolen, L, Phillips, R., and Lehman, M.N. 2012. Neural pathways by which leptin may modulate GnRH secretion in the sheep: activation of AgRP, POMC and kisspeptin (KNDy) neurons. 94th Annual Endocrinology Conference in Houston, TX. Abstract and poster presentation.

5. **Cernea, M**., Lee, T., Cheng, G., Padmanabhan, V., Lehman, M.N., and Coolen, L.M. 2011. Excess prenatal testosterone increases androgen receptor expression in hypothalamic areas of the female sheep brain. 41st Annual Society for Neuroscience Meeting in Washington, DC. Abstract and poster presentation.

6. **Cernea, M**., Padmanabhan, V., Merkley, C., Coolen, L.M., Lee, T., and Lehman, M.N. 2011. Effects of prenatal testosterone on KNDy-GnRH circuitry in a sheep model of PCOS. 93rd Annual Endocrinology Conference in Boston, MA. 2011. Abstract and poster presentation.

7. **Cernea, M**., Merkley, C., Coolen, L.M., Lee, T., Padmanabhan, V., and Lehman, M.N. 2011. Effects of prenatal testosterone on KNDy-GnRH circuitry in the sheep hypothalamus. The 1st Annual Michigan Alliance for Reproductive Technologies and Science (MARTS) Research Symposium, 2011. Michigan State University, Abstract and Poster.

8. **Cernea, M**., Merkley, C., Coolen, L.M., Lee, T., Padmanabhan, V., and Lehman, M.N. 2011. Effects of prenatal testosterone on KNDy-GnRH circuitry in the sheep hypothalamus. Anita Payne Lectureship and Poster Day, University of Michigan, 2010, Abstract and Poster.

RELEVANT WORK EXPERIENCE

2009-2010 **Teaching Assistant**
Anatcell 9531a: Neuroscience for Rehabilitation Sciences: two 2hr lectures per week, five 3hr labs in place of lecture, five 3 hr prep for lab including pre lab meetings, preparation of lab case studies and case study presentation).

PROFESSIONAL AFFILIATIONS

Society for Neuroscience Member (2010-Present)

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